Influences of Environmental Microbes on the Regulation of the Human Immune System in the Context of Allergies: From Epidemiology to Molecular Mechanisms

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Zusammenfassung

In den vergangenen Jahrzehnten haben die Fälle der am häufigsten vorkommenden Allergie, der IgE-vermittelten Hypersensitivität, zugenommen, so dass heute schon mehr als 25% der Schulkinder westlicher Länder darunter leiden. Eine Allegie wird ausgelöst, wenn eine B Zelle als Antwort auf Proteine aus der Umgebung IgE anstatt IgG sezerniert. Die Entstehung von allergischen Erkrankungen ist prinzipiell genetisch bedingt. Beobachtungen, dass Infektionenen oder Exposition gegenüber verschiedenen Umweltfaktoren wie Mikroben oder Diätfaktoren Kinder vor Allergien schützen, führten zur Formulierung der Hygiene-Hypothese. Diese Hypothese sagt, dass verminderte Exposition gegenüber infektiösen Substanzen während der frühen Kindheit zu abweichenden Immunantworten gegen harmlose Antigene im späteren Leben führt.

Das Ziel dieser Arbeit war zu untersuchen, wie Mikroben in der Umwelt das Immunsystem von Kindern beeinflusst, so dass sie vor Allergien geschützt sind. Wir verglichen die Expression von relevanten Markergenen des Immunsystems und untersuchten die Reaktionsfreudigkeit von Blutzellen von Bauern- und Nichtbauernkinder im Rahmen der Europäischen Querschnittsstudien ALEX und PARSIFAL. Im ersten Teil dieser Arbeit untersuchten wir, ob zwei oftmals vorgeschlagene immunologische Mechanismen für die Hygiene-Hypothese seine Relevanz auch für Bauernkinder hat. Beide Mechanismen, verschobenes Th-1/Th-2 Gleichgewicht, das den Immunoglobulin Klassenwechsel kontrolliert und die Bildung von regulativen T Zellen, konnten in unserem System nicht reproduziert werden.

Weil das angeborene Immunsystem die erste Verteidigungslinie gegen fremde Moleküle bildet und die Immunglobulin-vermittelte adaptive Immunantwort durch lösliche Faktoren steuert, vermuteten wir hier die Molekulare Grundlage für die Hygiene-Hypothese. Wir fanden, dass die Expression von Markergenen des angeborenen Immunsystems negativ mit dem Immunglobulin Klassenwechsel zu IgE assoziert war. Die Signalkaskade von Toll-like Rezeptoren, den wichtigsten Erkennungsrezeptoren des angeborenen Immunsystems, ist sehr genau reguliert, weil fehlerhafte Aktivierung zu Krankheiten wie Allergien und Autoimmunität führen könnte. Wir fanden, dass die Expression vom regulatorischen Molekül „Suppressor of cytokine signaling 1“ durch die Expostion gegenüber Mikroben in der Umwelt induziert wird und dass es so Kinder vor Allergien schützt. Aber nicht alle regulatorischen Moleküle der Signalkaskade von Toll-like Rezeptoren hatten dieselbe Relevanz in Kindern. Wir konnten diese Funktion nicht reproduzieren für ST2. Vielmehr schützte ST2 die Kinder gegen Allergien durch Regulation der T Helferzellaktivität.

Dann beschrieben wir MHC Klasse II Moleküle als neue Beteiligte im regulatorischen Netzwerk des angeborenen Immunsystems. Allerdings sind weiterführende Studien nötig um abzuklären, ob MHC Klasse II Moleküle auch am Schutz von Bauernkindern gegen Allergien beteiligt sind.

Schliesslich untersuchten wir, bei welchem Alter die Exposition gegenüber Mikroben in der Umwelt den grössten Schutz vor Allergien bringt und wie langer er anhält. Wir fanden heraus, dass vorgeburtliche Exposition den effektivsten Schutz vor Allergien bringt. Vorgeburtliche Exposition verändert die Expression von Markergenen des angeborenen Immunsystems anhaltend in Kindern und Mäusen. Es scheint, dass frühkindliche Exposition gegen Mirkroben bestimmt, wie das angeborene Immunsystem die Immunoglobulin-vermittelte adaptive Immunantwort im späteren Leben aktivert. Diese Veränderungen scheinen ausschlaggebend für den Schutz gegen allergische Erkrankungen zu sein.

Summary

The incidence of the most common form of allergy, the IgE-mediated hypersensitivity, has increased over the past decades and affects already more than 25% of school-age children of western countries. An allergy manifests through secretion of IgE instead of IgG by B cells in response to environmental proteins. The development of allergic disorders is principally regulated by genetic factors. Observations that infections or exposure to various environmental factors such as microbes or dietary factors protect children against the development of allergies, led to proposition of the hygiene hypothesis. This hypothesis states that decreased exposure to infectious agents early in life results in aberrant immune responses to otherwise innocuous antigens later in life.

The aim of this work was to investigate how exposure to environmental microbes influences children's immune system resulting in protection against allergies. We compared gene expression of relevant marker genes of the immune system and assessed responsiveness of blood cells of farmers' and non-farmers' children in the context of the European cross-sectional ALEX and PARSIFAL studies. In the first part of this work we investigated whether two often proposed immunological mechanisms for the hygiene hypothesis have relevance in farmers' children. Both mechanisms, a skewing of the Th-1/Th-2 balance controlling immunoglobulin class switching and the generation of T regulatory cells, could not be reproduced in our system.

Since the innate immune system constitutes the first contact to foreign molecules and directs the immunoglobulin-mediated adaptive immune response through release of soluble factors, we assumed here the molecular basis of the hygiene hypothesis. We found that the expression of marker genes of the innate immune system was negatively associated with immunoglobulin isotype switching to IgE. These marker genes, in turn, were induced through exposure to environmental microbes in children. Furthermore, the signaling cascade of Toll-like receptors, the primary pattern recognition receptors of the innate immune system, is tightly regulated, because miss-activation may lead to diseases such us allergy and autoimmunity. We found that the expression of the regulatory molecule suppressor of cytokine signaling 1 was controlled by environmental exposure to microbes and thereby protect children against the development of allergies. But not all of these regulatory molecules of the signaling cascade of Toll-like receptors had the same relevance in children. We failed the reproduce a regulatory function for ST2. By contrast, ST2 protected children against allergies via regulation of T helper cell activity.

Then, we identified MHC class II molecules as novel participant in the regulatory network of the innate immune response. Whether these molecules are involved in protecting children

living in environments containing higher amounts of microbes against allergies, has to be investigated in future studies.

Finally, we investigated the optimal age of exposure to microbial components to reach highest protection against allergies and how long it persists. It turned out, that prenatal exposure to microbes led to most effective protection of children against the disorders. Furthermore, prenatal exposure induced persistent modulation of the expression of marker genes of the innate immune system in children and in mice. Therefore, early-life exposure to microbial components shapes the way, how the innate immune system activates the immunoglobulin-mediated adaptive immune response in the later life. These modulations seem to be crucial to develop protection against allergic diseases.

CHAPTER 1

Introduction

Chapter Overview

This chapter gives an introduction in the epidemiology of IgE mediated allergic diseases and its increasing incidence in the past decades in western countries. Then, there is an introduction to the two arms of the immune system: the innate and the adaptive immunity. The tightly regulated innate immune system constitutes the first contact to foreign patterns and induces the B and T cell mediated adaptive immune system. Of the adaptive immunity, the T helper cell differentiation and the regulation of immunoglobulin class switching to IgE is considered profoundly. In the last part, an overview about the objectives of this study is given.

1. Epidemiology of allergic diseases

The development of allergic diseases is basically influenced by genetic factors[1]. Findings of regional variation in incidence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema in school-age children with the same genetic background made environmental components likely to be involved in the development of these diseases, too[2]. Children in developed countries like England or New Zeeland had about a 20 times higher prevalence of asthma symptoms than children of developing countries like Indonesia or Albania.

The incidence of allergic diseases was not always higher in western countries. The prevalence of asthma e.g. increased by 75% from 1980 to 1994 in the USA[3]. In Swedish schoolchildren the prevalence of asthma, hay fever, and atopic eczema doubled between 1979 and 1991[4]. Also other inflammatory diseases like Crohn's disease or autoimmunity have increased in developed countries. During the same period of time the incidence of infectious diseases like Hepatitis A, Mumps, or Tuberculosis has decreased in these countries[4-6].

2. The hygiene hypothesis

The term hygiene hypothesis has emerged to describe the correlation between the increase in hygienic conditions and the elevation especially in allergic disorders. The hygiene hypothesis was proposed after a lower risk of hay fever and atopic sensitization in children with more siblings was observed[7]. The protective effect was assigned to elevated infections during childhood. Other findings supported this hypothesis: early entry to day care of the children had a protective effect against the development of allergies[8] and Italian military students with antibodies to hepatitis A virus had a lower prevalence of atopy and atopic respiratory diseases[9].

Other studies showed that not only infections protected children against allergies, but also rural lifestyle[10]. Specially contact to farm animals seemed to be highly protective[11, 12]. The protective effect of the farm was assigned to exposure to higher loads of bacterial components as shown for endotoxin, a lipopolysaccharide of the outer layer of the cell membrane of Gram-negative bacteria[13, 14]. Furthermore, drinking of non-pasteurized milk or exposure of the mother to stable and stable animals during pregnancy had a protective effect on the children[15].

3. The ALEX and PARSIFAL studies

The goal of the international cross-sectional epidemiological studies ALEX (Allergy and Endotoxin)[16] and PARSIFAL (Prevention of Allergy Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle)[17] was to identify factors related to farm live and conferring protection of children against allergies and to identify the underlying immunological mechanisms. In the ALEX study, the bacterial cell wall component endotoxin was observed to correlate with protection of children against the development of allergies[13]. Maternal exposure to stable and stable animals during pregnancy were the most protective factors of the children of the PARSIFAL study (Chapter 6)[15]. Therefore, early life exposure to bacterial components seems to be important for providing effective protection. Furthermore, children spend blood to assess molecular mechanisms that are induced through exposure to microbial components and conferring protection against allergic diseases, i.e. constituting the immunological basis of the hygiene hypothesis. We assessed the expression of marker genes of the immune system in blood leukocytes and the responsiveness of these cells, the genetic background, and the immunoglobulin levels in serum of the children.

4. The immune system

The human immune system recognizes and eliminates invading microorganisms in two ways. The adaptive or acquired immunity, consisting of B and T cells, uses a vast set of antibodies and T cell receptors generated through gene rearrangement and somatic hypermutation with a high specificity against any foreign pattern or peptide. An organism needs 7 to 10 days to establish a fully running adaptive immune response. The highly effective acquired immunity is responsible for elimination of infection in a late phase and for the establishment of immunological memory. By contrast, the evolutionary conserved innate immunity constitutes the first line defense of a host to survive the early phase of infection. Furthermore, innate immunity provides co-stimulatory molecules and cytokines to direct the adaptive immune response. The innate immune system recognizes only a limited number of conserved structures of invading microorganisms through a limited number of germ-line

encoded pattern recognition receptors (PRR) and it seems to be independent of immunologic memory[18, 19].

4.1 The innate immune system

4.1.1 Pattern recognition receptors

Toll-like receptors

The Toll receptor was initially discovered in Drosophila. It is an evolutionary conserved receptor found in the worm Caenorhabditis elegans as well as in mammalians[18]. The immune system of the fruit fly is lacking an adaptive system making the Toll protein a central regulator of the immune defense. The signaling pathway of the receptor is a homologue to the one of the human innate immune system. It activates a NF-κB related transcription factor and induces production of antimicrobial peptides similar to the mammalian defensin family[20, 21].

In mammalian, 12 homologue forms of the Toll receptor are described and called Toll-like receptors (TLR), constituting the principal family of PRR in mammals. Humans have 10 functional TLR while TLR11 is non-functional due to a stop codon in the gene. Human TLR12 has not been documented so far. TLR are type I transmembrane glycoproteins[22]. The extracellular domain is composed of leucin-rich repeats (LRR), shaped as horseshoe structure and responsible for ligand binding[23]. The cytoplasmatic domain is homologous to that of the interleukin 1 receptor and termed Toll/IL-1R homology (TIR) domain[24].

TLR are mainly expressed on cells of the immune system such as macrophages, dendritic cells (DC), mast cells, B cells and some T-cells. Some TLR are found in endothelial cells, fibroblasts, adipocytes, and epithelial cells, too[25, 26]. The expression of TLR is not static but modulated in response to pathogens, cytokines, or environmental exposure to microbial components[27]. In chapter 6 and 7, we describe that especially exposure to microbial components in early life leads to persistent induction of TLR expression. Not all TLR are expressed extracellulary in the plasma membrane. TLR3, 7, 8, and 9 are found almost exclusively expressed in the endosomal compartments.

Based on their amino acid sequence, human TLR can be divided into five subgroups, each recognizing related structures[28, 29]. The ligand recognition concept of TLR is completely different to that of antibodies or T cell receptors generating a binding domain against almost any imaginable foreign structure. TLR rather recognize conserved molecules of microorganisms called pathogen-associated molecular patterns (PAMP).

TLR1, 2, 6, and 10. The TLR2 subfamily is composed of TLR1, 2, 6 and 10. This subgroup recognizes lipids of many different microorganisms (Table 1). TLR1 and 6 act as co-receptors of TLR2 recognizing lipoproteins of bacteria, mycoplasma, and mycobacteria[30-32]. TLR2 alone recognizes peptidoglycan and lipoteichoic acid from Gram-positive bacteria or lipoarabinomannan of mycobacteria (Figure 1). Together with the co-receptor dectin, TLR2 recognices zymosan from fungi[18, 28, 33]. The ligands of TLR10 are unknown.

TLR4. TLR4 is the TLR investigated in most detail. It recognizes lipopolysaccharides (LPS or endotoxin), an integral part of the outer cell membrane of Gram-negative bacteria (Table 1). LPS consists of an intracellular lipid A component and an extracellular oligosaccharide[34] (Figure 1). Even in picomolar range, LPS induces a strong inflammatory response in leukocytes, but also in platelets, endothelial cells, and epithelial cells[35], whereas the lipid A portion is the part carrying most biological activity. A single Escherichia coli cell contains about two millions LPS molecules. They are released upon cell death and during growth and division. In humans LPS is responsible, when entering the blood stream, for fever, hypotension, adult respiratory distress syndrome, disseminated intravascular coagulation, and endotoxin shock. By contrast, its presence in a low dose in the environment of children has been associated with protection against the development of allergies[13]. LPS is highly heat-stable; therefore a temperature of over 180°C is necessary to inactivate it[34]. Bacteria produce different LPS molecules. They vary in their phosphate patterns, number of acylchains, and fatty-acid composition reflecting the varied biological activities of different lipid A molecules, resulting in altered toxicity[18].

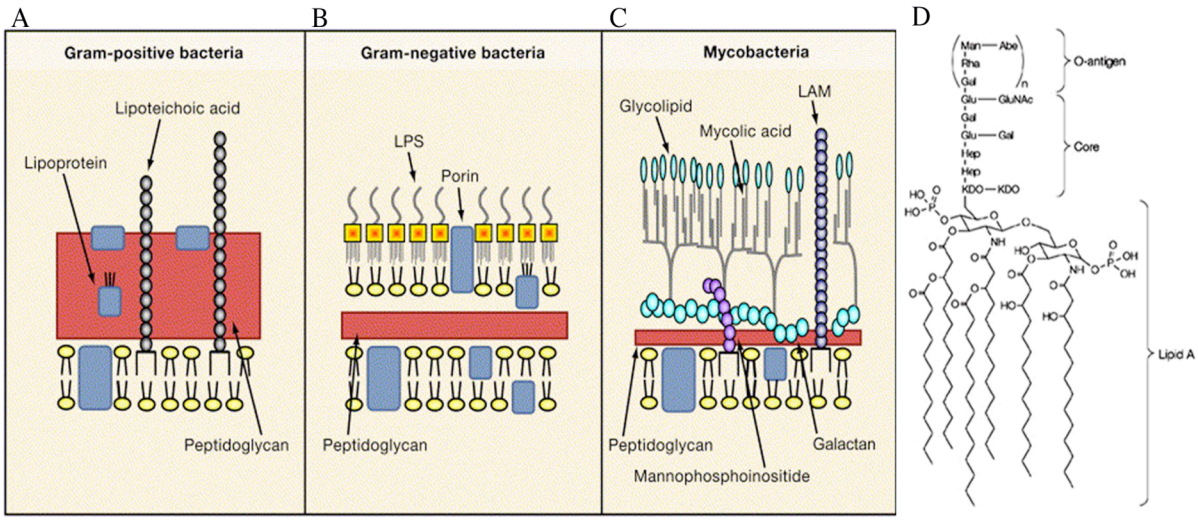

FIGURE 1. Overview of bacterial cell walls. A, The cell wall of Gram-positive bacteria consists of a thick layer of peptidoglycan. Lipoteichoic acids and lipoproteins are embedded in this cell wall. B, The cell wall of Gram-negative bacteria is characterized by a second lipid bilayer containing large amounts of LPS. C, Mycobacteria have a thick hydrophobic layer containing mycolyl arabinogalactan and dimycolate. Lipoarabinomannan is a major cell wall associated glycolipid. D, LPS is composed of the biological active lipid A (endotoxin), core oligosaccharide, and O-antigen. LPS, lipopolysaccaride; LAM, Lipoarabinomannan. (Adapted from Akira et al., Cell. 2006 Feb 24, 124 (4): 783-801 and from Miller et al., Nat Rev Microbiol. 2005 Jan, 3 (1): 36-46)

LPS binds to the LPS binding protein (LBP), an acute-phase protein present in serum. This complex binds to CD14, which exists in two forms. The soluble form (sCD14) is present in the blood plasma and the membrane bound form (mCD14) is attached to phagocyte membranes through a glycosylphosphatidylinositol (GPI) anchor. SCD14 enables stimulation of cells lacking the membrane bound form. LPS is transferred by soluble factor MD-2 to TLR4 leading to cell activation[18, 34, 36]. Other factors such as heat shock proteins (Hsp) 70 and 90, chemokine receptor (CXCR) 4, growth differentiation factor 5, CD11b, CD18, and CD55 are described to be involved in the LPS binding and cell activation complex[28, 37, 38].

Besides of LPS, TLR4 recognizes peptides, too. Examples are taxol from the Pacific yew having a potent anti-tumor activity in humans or endogenous ligands like Hsp 60 and 70. Heat shock proteins are induced in response to stressful condition or infection. It is thought that heat shock proteins activating macrophages and dendritic cells act as endogenous danger signal[28].

TLR5. TLR5 recognizes flagellin, the primary protein component of flagella (Table 1). Gram-negative bacteria use flagella as propellers to move through aqueous environment. Furthermore, flagella aid bacteria to attach to host cells and contribute thereby to the virulence of pathogenic bacteria[28].

TLR3. Double stranded (ds) RNA is produced by viruses. It is recognized by TLR3 and serves as potent inducer of type I interferons (INF-α and β), which have antiviral and immuno-stimulatory activities (Table 1). TLR3 is expressed on conventional DC and in a variety of epithelial cells functioning as barriers to infections. Another receptor for dsRNA is the dsRNA-dependent protein kinase (PKR) having immuno-stimulatory effects, too[18, 28].

TLR7, 8, and 9. Single stranded (ss) RNA is recognized by TLR7 and 8[39, 40]. Both genes are homolog to the TLR9 gene. TLR9 recognizes unmethylated CpG DNA, which is common in viral and bacterial genomes, but rare in vertebrate[18, 28] (Table 1).

Table 1. Toll-like receptors and their ligands

TLR	Ligands	Origin
TLR1	Tri-acyl lipopeptides	Bacteria, mycobacteria
TLR2	Lipoproteins	Bacteria, mycoplasma, mycobacteria
	Peptidoglycan	Gram-positive bacteria
	Lipoteichoic acid	Gram-positive bacteria
	Lipoarabinomannan	Mycobacteria
	Phenol-soluble modulin	Staphylococcus epidermidis
	Glycoinositolphospholipoids	Trypanosoma Cruzi
	Glycolipids	Treponema maltophilum
	Porins	Neisseria
	Atypical LPS	Leptospira interrogans
	Atypical LPS	Porphyromans gingivalis
	Zymosan	Fungi
	Heat-shock protein 70	Host
TLR6	Di-acyl lipopeptides	Mycoplasma
	Lipoteichoic acid	Gram-positive bacteria
	Zymosan	Fungi
TLR4	LPS	Gram-negative bacteria
	Taxol	Plants
	Fusion protein	Respiratory syncytial virus
	Envelope protein	Mouse mammary-tumour virus
	Heat-shock protein 60	Chlamydia pneumoniae
	Heat-shock protein 60	Host
	Heat-shock protein 70	Host
	Type II repeat extra domain A of fibronectin	Host
	Oligosaccharides of hyaluronic acid	Host
	Polysaccharide fragments of heparan sulfate	Host
	Fibrinogen	Host
TLR5	Flagellin	Bacteria
TLR3	Double stranded RNA	Virus
TLR7	Imidazoquinoline	Synthetic compounds
	Loxoribine	Synthetic compounds
	Bropirimine	Synthetic compounds
	Single stranded RNA	Virus
TLR8	Imidazoquinoline	Synthetic compounds
	Single stranded RNA	Virus
TLR9	CpG DNA	Bacteria, virus

Adapted from Takeda et al, Annu Rev Immunol 21, 335-76 (2003)

Other receptors of the innate immunity

NOD. Nucleotide-binding oligomerization domain (NOD) proteins are a family of 20 cytosolic molecules possessing a LRR domain for ligand recognition as well as caspase activation and recruitment domain (CARD) for induction of the downstream signaling cascade. NOD proteins act thereby as cytosolic PRR. Only ligands of NOD1 and NOD2 are known. They detect γ-D-glutamyl dipeptide and muramyl dipeptide found in bacterial peptidogycans. Binding of ligands to NOD1 and NOD2 causes their oligomerization and results in activation of NF-κB and of receptor-interacting protein (RIP) 2 kinase leading to release of inflammatory cytokines[18, 41]. Mutations in the NOD2 gene are associated with Crohn's disease, an inflammatory bowel disease associated with enhanced NF-κB activity and T helper cell type 1 response[42, 43].

TREM. Triggering receptors expressed by myeloid cells (TREM)1 and TREM2 are transmembrane glycoproteins consisting of a single extracellular immunoglobulin-like domain, a transmembrane region with charged lysine residues, and a short cytoplasmatic tail. Both receptors associate with DAP12 for signaling and function. DAP12 contains an immunoreceptor tyrosine-based activation motif (ITAM), which after phosphorylation, recruits tyrosine kinases ζ-chain-associated protein 70 (ZAP70) and spleen tyrosine kinase (SYK). The signaling cascade ends in the activation of phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ1, and p44-p42 extracellular-signal-regulated kinase (ERK). Triggering of this pathway leads to Ca^{2+} release, rearrangement of the actin cytoskeleton, and activation of transcriptional complexes. Human TREM1 is expressed by blood neutrophils, monocytes, and macrophages. The expression is induced in neutrophils and epithelial cells during an infection with bacteria or fungi. TREM1 amplifies TLR-initiated inflammatory responses to microbial challenges. Although the ligand of TREM1 is unknown, it is clear that TREM1 has an important role in establishing inflammation and endtoxin shock[44, 45]. Human TREM2 is expressed by immature DC. After activation, DC down-regulate the expression of TREM2. TREM2 seems to be involved in DC maturation as well as in the maturation of the non-immunological osteaclasts, microglia, and oligodendrocytes[45].

RIG-I and Mda5. Retinoic-acid-inducible protein (RIG)-I and melanoma differentiation associated gene (Mda) 5 are cytosolic RNA helicases carrying 2 caspase activation and recruitment domain (CARD)-like domains. The RNA helicases recognize viral dsRNA and the CARD-like domains are responsible for activation of downstream signaling pathways leading to activation of transcription factors NF-κB and interferon regulatory factor (IRF)-3, thereby inducing inflammatory cytokines and type I IFNs[18, 29, 46].

4.1.2 TLR signaling pathway

After ligand binding, TLR dimerize and undergo conformational changes. This enables the recruitment of TIR-domain containing adaptor molecules to the TIR domain of the TLR. There are five such adaptor molecules known: Myeloid differentiation primary-response protein 88 (MyD88), TIR-associated protein (TIRAP/MAL), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF/TICAM1), TRIF-related adaptor molecule (TRAM), and steril α and armadillo motifs (SARM). Alternative usage of these adaptor molecules by TLR explains the differing responses induced by different TLR ligands[18, 42, 47]. SARM negatively regulates adaptor protein TRIF dependent TLR signaling[48].

MyD88 dependent signaling pathway

The MyD88 dependent signaling pathway is homologous to the one of the IL-1 receptor. MyD88 has a C-terminal TIR domain, which associates with the TIR domain of TLR after ligand binding, and an N-terminal death domain. TLR2 and TLR4 require another adaptor protein, TIRAP, for MyD88 recruitment. The IL-1R-associated kinase (IRAK)-4 is recruited to MyD88 via dimerization of the death domains of the molecules. IRAK-4 then activates IRAK-1 through phosphorylation. IRAK-1 subsequently associates with TNFR-associated factor (TRAF) 6 an ubiquitin protein ligase E3. TRAF6 catalyses polyubiquitination of itself and of NEMO (IKK-γ/NF-κB essential modulator) supported by the E2 ubiquitin ligase complex composed of UBC13 and UEV1A. Another complex composed of TGF-β-activated kinase 1 (TAK1) and TAK1 binding proteins (TAB) 1-3 is recruited to TRAF6, too. TAK1 activates two different signaling ways, the MAP kinase cascade leading to activation of the AP-1 complex (p38 kinase and Jun N-terminal kinase (JNK)) and the activation of the IκB kinase (IKK) complex including NEMO, IKK-γ, IKK-α, and IKK-β. Activated IKK complex induces phosphorylation and ubiquitination of IκB ending in its degradation by the proteosome. Liberated transcription factor NF-κB and AP-1 complex translocate to the nucleus to induce transcription of inflammatory genes[18, 42]. The transcription factor IRF-5 is also activated by TRAF6 and is responsible for induction of cytokine genes[49] (Figure 2).

TRIF dependent signaling pathway

TLR3 and TLR4 have the ability to induce type I IFN (IFN-α and IFN-β) in a MyD88 independent way. Instead of MyD88, the adaptor TRIF is recruited to the receptor after ligand binding. TLR4 needs support of TRAM to bind TRIF. TRIF interacts with the receptor-interacting protein 1 (RIP1), which activates NF-κB but with delayed kinetics compared to the MyD88 dependent way[50]. Furthermore, TRIF activates TRAF-family-member-associated NF-κB activator (TANK) binding kinase 1 (TBK1) leading to phosphorylation and

translocation of the transcription factors IRF-3 and IRF-7, responsible for induction of IFN-inducible genes. TLR7 and TLR9 ligands are also able to induce type I IFN production in a TRIF independent, but MyD88 dependent way[18, 42] (Figure 2).

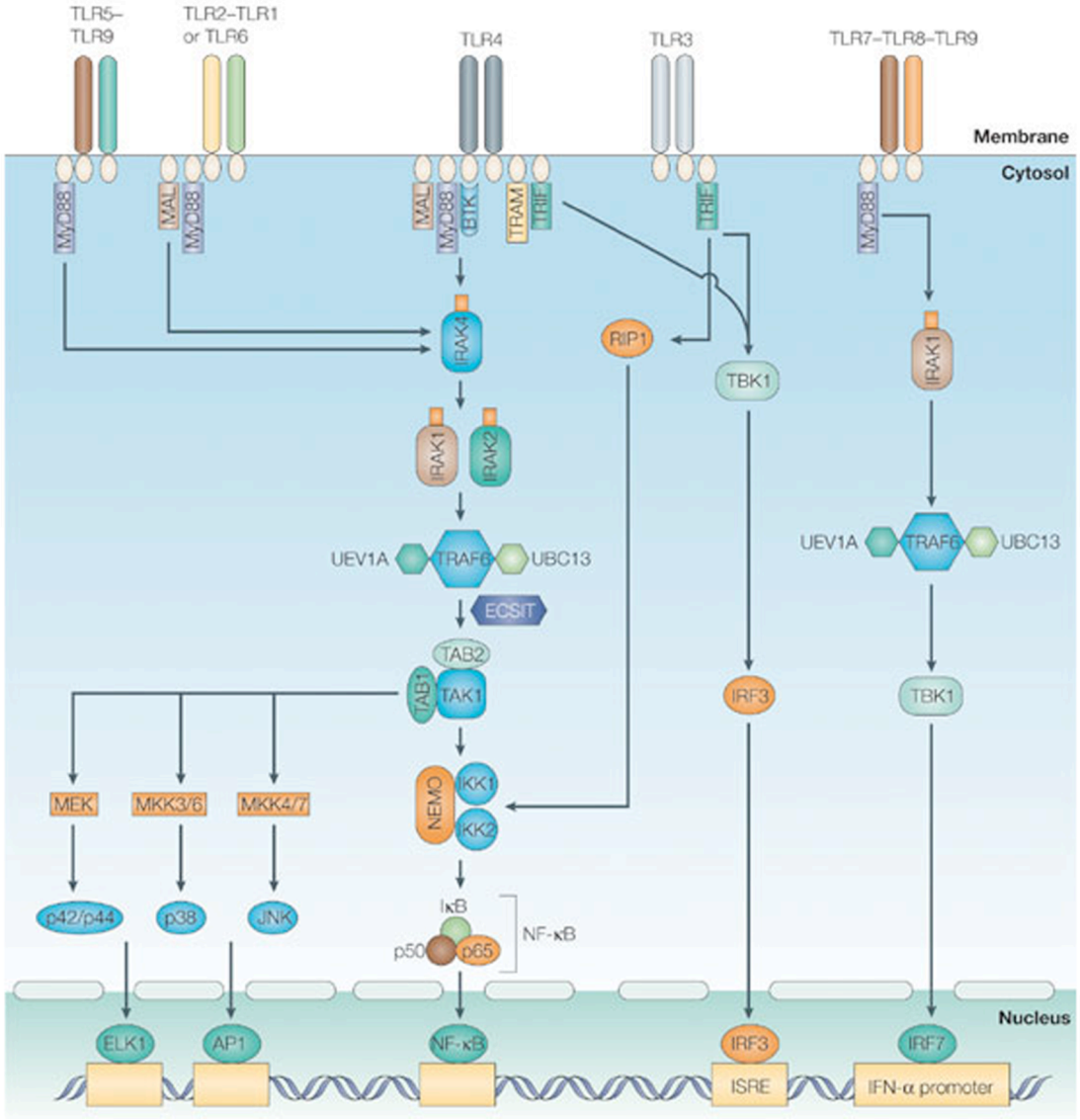

FIGURE 2. Toll-like receptor signaling cascade. MyD88 dependent pathway: MyD88 is recruited to the receptor after ligand binding. Then, MyD88 binds the kinase IRAK-4, which activates IRAK-1. IRAK-1 associates with TRAF6, which is responsible for polyubiquitination of NEMO and activation of the complex of TAK1 and TAB1-3 leading to translocation of NF-κB into the nucleus and to activation of MAP kinase. TRIF dependent pathway: TLR3 and TLR4 have the ability to recruit TRIF to the receptor after ligand binding. TRIF interacts with RIP1 leading to NF-κB translocation and with TBK1 inducing translocation of transcription factors IRF-3 and IRF-7 into the nucleus ending in induction of type I IFN. MyD88, myeloid differentiation primary-response protein 88; IRAK, IL-1R-associated kinase; TRAF6, TNFR-associated factor 6; NEMO, IKK-γ/NF-κB essential modulator; TAK1, TGF-β-activated kinase 1; TAB, TAK1 binding proteins; TRIF, TIR-domain-containing

adaptor protein-inducing IFN-β; RIP, receptor-interacting protein; IFN, interferon. (Adapted from Liew et al., Nat Rev Immunol. 2005 Jun, 5 (6): 446-58)

4.1.3 Regulators of the TLR signaling cascade

Activation of the TLR signaling cascade is absolutely necessary to establish an immune response. But the process has to be tightly regulated, because misguided activation of the innate immune system may enhance the risk to develop chronic inflammation, allergy, and autoimmunity[1].

Repeated challenge with LPS leads to a reduced responsiveness to a subsequent stimulation with LPS. This is a well known phenomenon and is designated as endotoxin or LPS tolerance[42]. Many different molecules and mechanisms have been proposed to be involved in negative regulation of the TLR signaling cascade. We investigated two of these molecules, SOCS-1 and ST2, whether they have regulatory function in children exposed to high levels of endotoxin in their environment and whether these molecules may protect them against allergic diseases (Chapter 3 and 4).

Regulation starts in blood and tissues, where soluble TLR interact with ligands, and ends in many intracellular regulators controlling the TLR signaling cascade (Figure 3). Regulation also occurs through down-regulation of transcription and translation of TLR genes or by degradation of TLR protein[51]. Furthermore, we have identified MHC class II molecules as novel participants in the complex regulatory mechanisms that control the efficiency of innate immune responses (Chapter 5).

Soluble TLR. Soluble forms of TLR2 and TLR4 have been found in mammalians. Soluble TLR2 has been described in humans, whereas soluble TLR4 has been found in mice. The mechanism by which the soluble form attenuates TLR signaling is unknown. However, soluble forms of TLR might serve as important first-line negative regulators of innate immunity[51].

MyD88s. MyD88s is an alternative splice variant of MyD88 lacking the intermediate domain situated between the death domain, which mediates contact to the IRAK family, and the TIR domain interacting with TLR. MyD88s is induced by LPS in monocytic cells and inhibits LPS-induced NF-κB activation. MyD88s inhibits the ability of IRAK-4 to phosphorylate IRAK-1 so that IRAK-4 cannot be recruited to the receptor complex[51-53].

SOCS. The family of suppressor of cytokine signaling (SOCS) proteins is well described as key regulator molecule in TLR signaling. The human family consists of seven members[54-56]. SOCS-1 and SOCS-3 were originally found to be necessary in suppressing inflammatory

responses by acting as negative feedback inhibitor in cytokine signaling pathways by inhibiting the JAK kinase activity[57-59]. Mice lacking SOCS-1 die within 3 weeks because of organ damage induced by excessive levels of INF-γ[60]. At least some of the SOCS proteins (SOCS-1 and SOCS-3) are induced through TLR agonists such as LPS or unmethylated CpG DNA[61, 62]. SOCS proteins negatively regulate TLR signaling through induction of degradation of TIRAP[63]. We found that SOCS-1 is up-regulated in children living in an environment rich in microbes. Furthermore, expression of SOCS-1, in turn, correlates with decreased secretion of the inflammatory cytokine INF-γ and reduced IgE levels (Chapter 3).

IRAK-M and IRAK-2. The expression of IRAK-M is restricted to monocytes and macrophages and it is induced through TLR ligands. IRAK-M has no kinase activity and it prevents the dissociation of the IRAK-1 – IRAK-4 complex from MyD88. IRAK-M decreases the response to TLR stimuli and is required for endotoxin tolerance[51, 52, 64]. The IRAK-2 gene consists of four splice variants called IRAK-2a-d. IRAK-2c and IRAK-2d are missing the death domain. They are induced through LPS and inhibit LPS-induced NF-κB activation[51].

PI3K. PI3K is a heterodimeric protein consisting of a p85 regulatory subunit and a p110 catalytic part. It is constitutively expressed by most cells and functions as signal in many cellular events. P85-deficent mice show enhanced IL-12 production in response to TLR ligands, possibly because of increased activation of the p38 kinase[51, 52]. Other groups found that PI3K has a positive role in signaling process as inducer of NF-κB activation[65]. The discrepancy may be due to differences in cell types used.

TOLLIP. TOLLIP (Toll-interacting protein) interacts in resting, unstimulated cells with IRAK-1, thereby preventing NF-κB activation by blocking phosphorylation of IRAK-1. After TLR stimulation, the IRAK-1 – TOLLIP complex is recruited to the receptor leading to autophosphorylation of IRAK-1 and its dissociation from the receptor. Furthermore, IRAK-1 phosphorylates TOLLIP ending in its ubiquitylation and degradation. Therefore, TOLLIP maintains the immune cell in a quiescent state in absence of stimulation[51, 52, 66].

A20. The expression of A20 is induced by LPS. A20-deficient macrophages produce elevated levels of pro-inflammatory cytokines following stimulation of TLR. A20 is a cysteine protease deubiquitylating enzyme that blocks TLR signaling by deubiquitylating TRAF6[51].

TRIAD3A. TRIAD3A is an E3 ubiquitin-protein ligase, down-regulating the protein expression of TLR4, TLR9, and to a minor degree of TLR3 and TLR5. Degradation by ubiquitination is a general mechanism to regulate protein expression. Covalent attachment of ubiquitin to the target protein is a pathway with three enzymes involved. The E1 ubiquitin-activating protein activates ubiquitin by formation of a thioester bond between ubiquitin and

cysteine at the active site of E1. Ubiquitin is then transferred to a second ubiquitin-conjugated enzyme (E2) to form a thioester linkage. E3 promotes formation of an isopeptide bond between ubiquitin and lysine residues on the target protein ending in degradation of the ubiquitinated protein by the proteosome[67].

SHIP. SHIP expression is induced though LPS and mediates increase in TGF-β production by macrophages and mast cells. SHIP inhibits PI3K mediated NF-κB activation and is thereby essential for endotoxin tolerance[68].

RIP3. RIP3 negatively regulates TRIF signaling by preventing association between RIP1 and TRIF. Thereby, it inhibits RIP1 mediated NF-κB activation[50, 69].

ST2. ST2 (also known as T1, Fit-1 or DER4) is a type I transmembrane protein with three extracellular immunoglobulin-like domains and an intracellular TIR domain[70, 71]. A soluble form of ST2 exists, too. It is generated by alternative splicing and is present in normal human serum. ST2-deficient mouse macrophages produce elevated amounts of cytokines after stimulation of TLR2, TLR4, and TLR9, but not of TLR3. ST2 achieves its inhibitory effects through sequestration of MyD88 and TIRAP[72-74]. After 4 hours *in vitro* stimulation with the TLR4 stimulus LPS or with inflammatory cytokines, ST2 is slightly induced in macrophages or monocytes turning ST2 into a negative feedback inhibitor[74, 75]. Soluble ST2 is increased in serum of patients with inflammatory diseases like asthma but also with autoimmunity, indicating an influence of ST2 on T helper cell differentiation[76-78]. We found that ST2 expression is decreased in children exposed to microbial components and this, in turn, is associated with reduced T helper cell activation, lower IgE levels in serums, and fewer incidences of allergic diseases. In children living in areas containing high levels of microbial components, ST2 fails to act as negative regulator of the TLR signaling cascade (Chapter 4).

SIGIRR. SIGIRR is a transmembrane protein with a single extracellular immunoglobulin domain and a cytoplasmatic TIR domain. It is expressed on epithelial cells, immature dendritic cells, but not on macrophages. Mice lacking SIGIRR have an increased susceptibility to endotoxin shock and DC of these mice show an elevated response to TLR4 and TLR9 ligands. The mechanism by which SIGIRR suppresses TLR function is probably via interaction with TLR4, IRAK, and TRAF6[51, 79].

TRAILR. TRAILR belongs to the TNF superfamily and is the receptor of TRAIL. Stimulation through TLR2, TLR3, and TLR4, but not TLR9 leads to enhanced expression of TRAIL and to elevated production of cytokines in TRAILR-deficient macrophages. TRAILR seems to inhibit TLR signaling by stabilizing the IκB complex and thereby inhibiting nuclear translocation of NF-κB[51].

RP105. RP105 is a TLR-like molecule, lacking the cytoplasmatic TIR domain. It is expressed in B-cells and antigen-presenting cells (APC) and acts as specific inhibitor for TLR4 signaling. RP105 forms a complex with MD-1 and directly interacts with the TLR4 – MD-2 complex inhibiting the ability of this complex to bind LPS[80].

Apoptosis. Interaction of TLR2 with its ligands can trigger apoptosis in macrophages and epithelial cells. The process seems to be dependent on MyD88, FADD (Fas-associated via death domain), and caspase-8 inducing the apoptotic cascade. However, the activation of NF-κB through MyD88 is anti-apoptotic. It is not clear, how positive and negative regulation of apoptosis though the same TLR signaling pathway is regulated on cellular levels[30, 51].

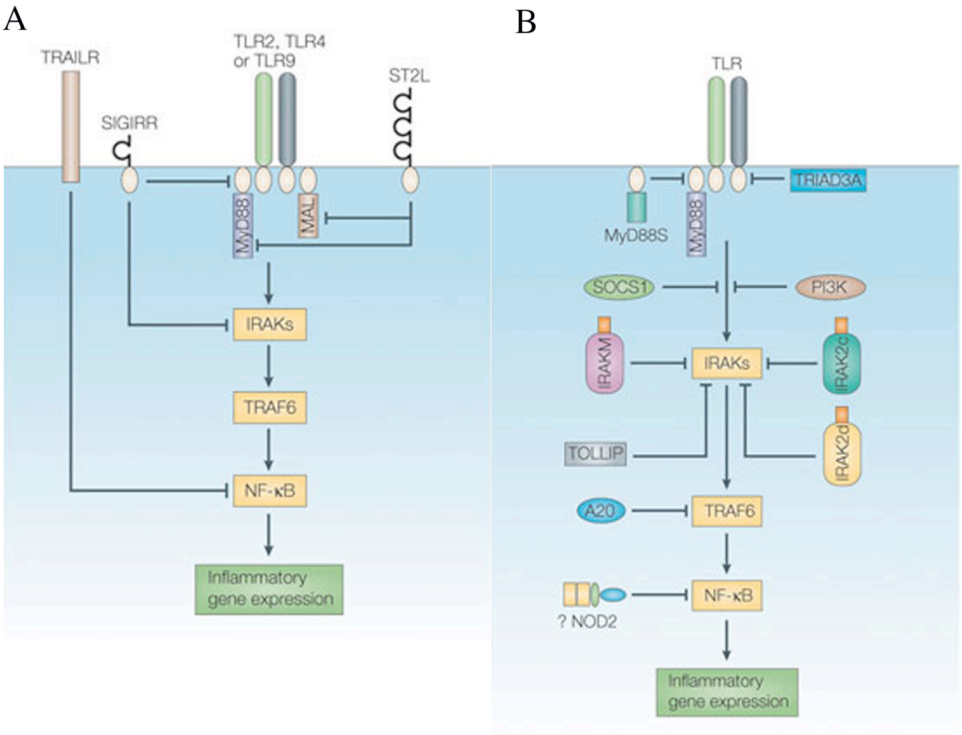

FIGURE 3. Regulators of Toll-like receptor signaling cascade. A, Transmembrane regulators of TLR signaling cascade: ST2 and SIGIRR inhibit MyD88 dependent NF-κB activation through binding to MyD88, TIRAP or IRAK. TRAILR supress NF-κB activation in a later stage of the TLR signaling cascade. B, Intracellular regulator of TLR signaling cascade: TRIAD3A induces polyubiquitination and degradation of certain TLR, while MyD88s antagonizes MyD88 functions. Regulatory molecules like SOCS1, IRAK-M, TOLLIP, IRAK2c and IRAK2d selectively suppress TLR signaling on various stages. PI3K negatively regulates some TLR responses. A20 deubiquitylates TRAF6 and affects MyD88-dependent and MyD88-independent pathways. MyD88, myeloid differentiation primary-response protein 88; TIRAP, TIR-associated protein; IRAK, IL-1R-associated kinase; MyD88s, short form of MyD88; SOCS-1, suppressor of cytokine signaling 1; TOLLIP, toll-interacting protein; PI3K, phosphatidylinositol 3-kinase; TRAF6, tumour-necrosis factor-receptor-associated factor 6. (Adapted from Liew et al., Nat Rev Immunol. 2005 Jun, 5 (6): 446-58)

4.2 Inflammation

Inflammation is observed in infections, but also in autoimmunity, allergies, atherosclerosis, or Alzheimer's disease. It is characterized by swelling, pain, increase in body temperature, tissue injury, and the influx of immunologically active cells. During an inflammatory response a variety of mediators are released by cells of the innate and adaptive immune systems. These cells are tissue mast cells, blood platelets, neutrophiles, monocytes, macrophages, eosinophils, basophils, and lymphocytes. After an infection, the innate immunity initiates an inflammatory response with the aim of fighting the invading pathogens in the early phase of infection, to attract immune cells to the host of infection, and to activate an adaptive immune response.

Challenging innate immune receptors leads to translocation of transcription factors to the nucleus and to activation of MAP kinases. These molecules induce a wide spectrum of inflammatory responses such as induction of cytokines and chemokines (e.g. tumor necrosis factor (TNF), IFN-γ, or IL-1, IL-6, IL-8, IL-12 [47, 81, 82]), NO production[83], expression of antimicrobial peptides (e.g. human β-defensin (hBD)-2[84]), apoptosis[30], dendritic cell maturation including up-regulation of major histocompatibility complex (MHC) molecules and co-stimulatory molecules like CD80 and CD86, two signals necessary for T cell activation[85, 86].

Other systems producing inflammatory mediators are the kinin system, the clotting system, the fibrinolytic system, and the complement system. The kinin system is activated following tissue injury and produces potent vasoactive mediators that increase vascular permeability, cause vasodilation, induce pain, and contraction of smooth-muscle. The clotting system is triggered by damage to blood vessels and prevents bleeding and limits the spread of invading pathogens into the bloodstream, but it increases the vascular permeability and neutrophil influx. The fibrinolytic system removes fibrin clots from injured tissues. Degradation products are chemotactic for neutrophils. Finally, the complement pathway results in formation of complement split products that serve as important mediators of inflammation. These mediators induce smooth-muscle contraction and increase vascular permeability. Neutrophils and monocytes migrate towards the site of complement activation in tissues. The complement system is composed of more than 20 serum proteins and cell surface receptors, which assemble to a membrane attack complex that introduces pores into the membrane of invading pathogens such as bacteria[87].

Following membrane perturbations several cell types like macrophages, monocytes, neutrophiles, and mast cells produce prostaglandins, thromboxanes, and leukotrienes. These lipid inflammatory mediators induce smooth-muscle contraction and are potent chemoatractants of neutrophils.

4.3 The adaptive immune system

4.3.1 T helper cell activation

Naïve $CD4^+$ T helper (Th) cell activation is initiated by interaction of the T cell receptor (TCR) - CD3 complex with a processed antigenic peptide bound to MHC class II molecules presented on antigen-presenting cells (APC). The TCR dictates the antigen specificity of the response and plays the central role in initiating T cell activation[88]. Released cytokines and co-stimulatory molecules of activated Th cells control the adaptive immunity. These cytokines induce B cell proliferation and determine the isotype of antibodies secreted by B cells and thereby the effector function of the adaptive immune response.

MHC class II molecule. MHC class II molecules are membrane bound glycoproteins constituting of one α and one β polypeptide chain encoded by multiple α and β chain genes. Three isotypes of human MHC class II molecules exist, which are named according to the corresponding gene regions HLA-DR, HLA-DQ, and HLA-DP[89]. MHC class II molecules have two external domains built by the α_1 and α_2 domains of one chain and the β_1 and β_2 domains of the other chain. The α_1 and β_1 form the highly polymorphic peptide binding cleft. MHC class II molecules are primarily expressed on thymic epithelial cells, activated T cells, and APC such as B cells, monocytes/macrophages, and DC. Exogenous antigens are processed in endocytic vesicles and loaded on MHC class II molecules. Resulting peptide - MHC class II complexes move to the plasma membrane. Expression of MHC class II molecules is regulated through class II transactivator (CIITA). The structure of CIITA is homologous to the NOD proteins because of the C-terminal LRR for ligand binding, the NOD, domain and the CARD transcription enhancer domain. Furthermore, CIITA has a N-terminal transcription activator domain, enabling CIITA to interact with co-factors for induction of transcription. CIITA itself does not bind DNA, but it associates with four DNA-binding factors: regulatory factor X (RFX) 5, RFX-associated protein (RFXAP), RFX-associated ankyrin-containing protein (RFXANK), and the X2-box-binding factor cyclic-AMP-responsive-element-binding protein (CREB). Ligands interacting with CIITA are not known. CIITA transcription is up-regulated through IFN-γ. Furthermore, there is a number of genes that are repressed by CIITA, including those encoding IL-4 and IL-10[90-93].

T cell receptor. The TCR is a heterodimer constituting of an α and a β chain or a γ and a δ chain. TCR composed of α and β chain is much more abundant than the γ/δ-TCR. The extracellular domain is folded in two immunoglobulin domains consisting of a constant (C) and a variable (V) region, structurally homologous to the variable and constant domains of immunoglobulin. Both chains of TCR have a positively charged transmembrane region and a short cytoplasmic tail. The positively charged transmembrane region enables heterodimer

formation with the negatively charged chains of the signal-transducing CD3 complex. TCR is expressed after immature T cells migrated from bone marrow to the thymus[88].

The α chain of the TCR is encoded by V, J, and C gene segments on germ-line DNA, the β chain by V, D, J, and C gene segments. Rearrangement of α and β chain gene segments results in VJ joining for the α chain and in VDJ joining for the β chain. V, D, and J gene segments are flanked by recognition signal sequences (RSS) recognized by recombinase enzymes recombination-activating genes (RAG-1 and RAG-2) catalyzing the V-J and V-D-J joining. The diversity of the TCR is achieved by combinatorial joining of variable-region gene segments generating a large number of random gene combinations. Diversity is further increased by alternative joining of D gene segments in different numbers yielding VDDJ or VDDDJ segments. Junctional flexibility, P-region nucleotide addition, and N-region nucleotides further enhance the diversity of the TCR considerable[88].

After T cell rearrangement, immature thymocytes undergo a positive selection resulting in MHC restriction. Those cells survive that carry a TCR capable of binding self-MHC molecules. In the negative selection process, all T cells expressing high affinity receptors to self-MHC molecules alone or self-antigen presented by self-MHC are eliminated, a process resulting in self-tolerance. The mature T cell migrates to the periphery, ready to get activated. However, the signal through the TCR is not sufficient to fully activate a naïve T cell. A co-stimulatory signal provided by the interaction of CD28 of the T cell and CD80/CD86 of the APC is necessary, too[88].

T helper cell activation and differentiation. APC are central to T lymphocyte activation. APC take up antigens in peripheral tissues, become activated and migrate to lymphoid tissues to present the antigenic peptides on MHC class II molecules. Depending on the density of the peptides presented, types of co-stimulatory molecules expressed and cytokines released by the APC, naïve T helper cells start to proliferate and differentiate into T effector cell subsets, T helper cell type (Th) -1, Th-2, T regulatory cell (Treg), and Th-17 (Figure 4). The activation of $CD8^+$ cytotoxic T lymphocyte (CTL) requires similar signals, except peptides must be presented by MHC class I molecules and memory CTL need additional signals of the T helper cells to become activated. The principal pathway, by which APC become activated to provide the signals to naïve T cells occurs via TLR recognition of pathogens[94].

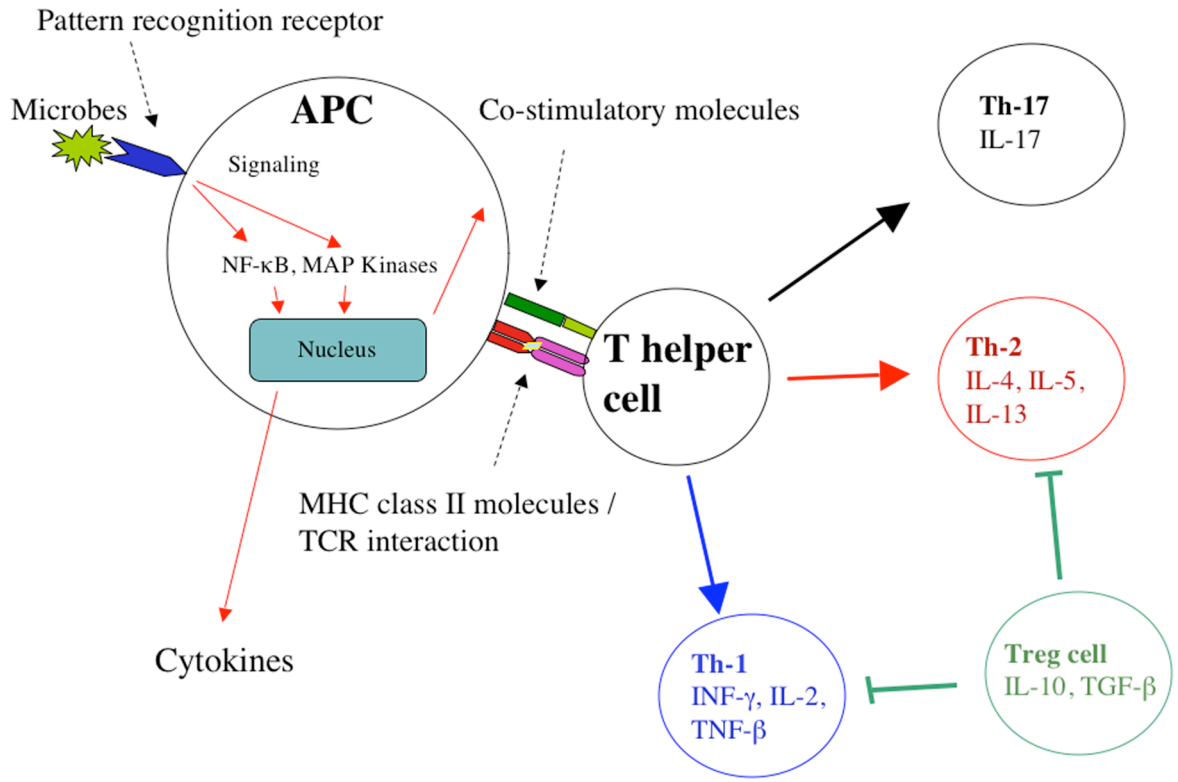

FIGURE 4. Activation of T helper cells by antigen-presenting cells. APC become activated through engagement of antigenic structures by a receptor of the innate immunity (PRR) leading to up-regulation of co-stimulatory molecules and secretion of cytokines. Furthermore, foreign molecules are internalized, processed, and short antigenic peptides are presented on MHC class II molecules to the TCR of a T helper cell. All three signals induce T helper cell activation and differentiation. T helper cell differentiation is determined by the type of cytokines present and by the strength of the activating signals. APC, antigen-presenting cell; Th, T helper cell; Treg, T regulatory cell; TCR, T cell receptor.

TLR-induced IL-12 production of APC generally induces differentiation towards Th-1 cells. Immunization of mice with high dose of TLR ligands or complete Freund's adjuvant results in Th-1 response. Furthermore, mice lacking MyD88 show impaired Th-1 but normal Th-2 responses[94-96]. By contrast, there are studies describing a Th2 response after triggering TLR2[94, 97, 98]. Finally, low dose of inhaled LPS induces a Th-2 response, while high dose of LPS induces a Th-1 response in mice[99]. We found in chapter 2 that exposure to environmental microbial components of children fail to skew the T helper cell balance. Other factors that influence the T cell differentiation are the notch ligands, jagged1 and delta1 expressed on APC. Delta1 interaction with notch3 leads to Th-1 differentiation, while jagged1 interaction with notch1 mediates differentiation in Treg cells[100-102].

Th-1 response is characterized by the secretion of IL-2, TNF-β, and IFN-γ induced by transcription factor T-bet[103]. IFN-γ mediates secretion of high affinity IgG2a antibodies in B

cells and activates macrophages but inhibits Th-2 cells. Dysregulation of the Th-1 response is associated with autoimmunity and inflammatory diseases.

Th-2 cell differentiation, evolved to enhance clearance of parasites, is induced by IL-4. Th-2 cells express transcription factor GATA-3 enhancing the secretion of IL-4, IL-5, and IL-13[104]. These cytokines activate eosinophils, mast cells, and mediate IgE secretion in B cells, but inhibit Th-1 cells. Dysregulated Th-2 response is associated with allergies and asthma.

Treg cells act as suppressor for Th-1 and Th-2 cells and are therefore important regulators of the immune response. The suppression depends, at least in part, on cell to cell contact[105]. There are two subgroups of Treg cells, antigen-specific Treg cells and non-specific or naturally occurring Treg cells. Furthermore, Treg cells are characterized through expression of the transcription factor FOXP3 and secrete elevated amounts of the cytokines IL-10 and TGF-β[106, 107]. Since these cells should not interfere with induction of pathogen-specific protective immune response, TLR-mediated IL-6 production of APC blocks the suppressive activity of Treg cells[94, 108]. Furthermore, stimulation of TLR8 bearing Treg cells by TLR ligand reverses their suppressive function[109]. By contrast, direct TLR4 challenge of Treg cells with high concentrations of LPS elicits their proliferation, prolongs their survival, and augments their *in vitro* suppressive activity[110, 111].

Th-17 cells are induced through TGF-β and IL-6 and produce high amounts of IL-17. IL-23 is important for amplifying and stabilizing the Th17 phenotype. The development of Th-17 cells is blocked by IL-4 and IFN-γ and it seems that these cells have a function in suppressing autoimmune disease[112, 113].

4.3.2 B cell activation

B cells produce antibodies, a most effective tool of the immune system to fight infections. B cells mature in the bone marrow. During maturation, the process of gene rearrangement of B cell receptors (membrane bound form of antibody) takes place to generate antibodies with a vast diversity of binding specificities. Mechanistically, the process is quite similar to the rearrangement of the TCR genes. The antibody light chain gene consists of a V, J, and C gene segment, while the antibody heavy chain gene contains a V, D, J, and C gene segment. Antibody diversity is generated by multiple germ-line gene segments, combinatorial V-(D)-J joining, junctional flexibility, P-region and N-region nucleotides addition, and combinatorial association of light and heavy chains.

After maturation, B cells bearing the immunoglobulin (Ig, antibody) isotype M on the cell surface migrate to peripheral lymphoid organs, where they get activated through antigenic contact. Further signals required for B cell activation come from the T helper cell. The

interaction of CD40 with CD40 ligand on the T helper cell provides the second activation signal. The third signal comes from cytokines secreted by T helper cells.

Immunoglobulin class-switch recombination. After immunization with antigen, B cells proliferate for about a week. Then, they migrate to germinal centers of secondary lymphoid organs, where somatic hypermutation and class-switch recombination (CSR) take place. Via somatic hypermutation, point mutations are induced in the variable region of the antibody gene to generate antibodies with enhanced specificity. B cells bearing receptors with the highest affinity to the antigen are selected by affinity maturation. By contrast, CSR exchanges the constant region of the antibody, generally Cμ (IgM) by Cγ (IgG), Cα (IgA), or Cε (IgE) (Figure 5). CSR changes the antibodies' effector function. Both processes have in common that they occur in antigen-stimulated B cells, require transcription through the target region and require the enzyme AICDA (activated B cell specific enzyme activation-induced cytidine deaminase). But CSR has no well-defined consensus target sequence[114] and does not necessarily have to take place in lymph nodes[115].

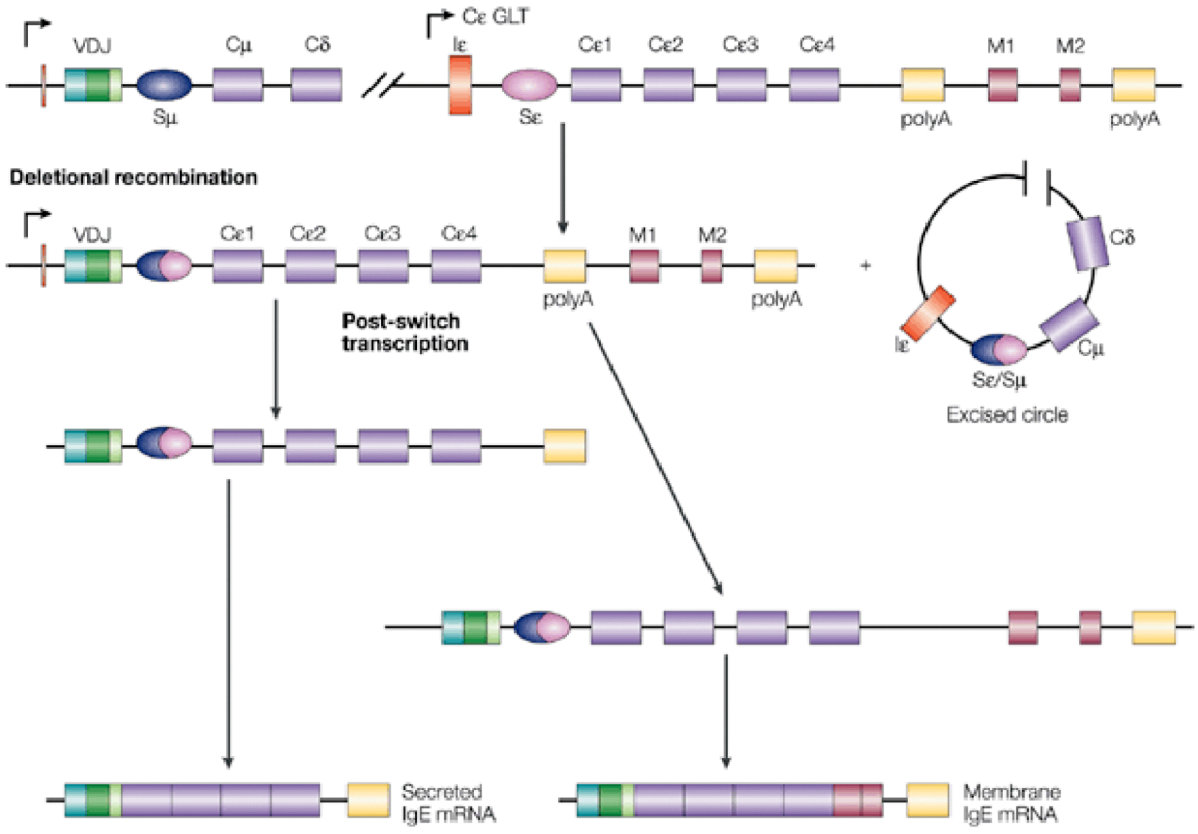

FIGURE 5. Immunoglobulin class-switch recombination. Immunoglobulin class-switch recombination takes place a few days after induction of proliferation of B cells changing the effector funtion of an antibody. During CSR a large piece of DNA is excised. The process is initiated after transcription of germ-line transcripts starting at the I promotor generating a single stranded R-loop structure accessible for CSR recombinase AICDA. GLT, germ-line transcript; CSR, class-switch recombination; AICDA,

activation-induced cytidine deaminase. (Adapted from Geha et al., Nat Rev Immunol. 2003 Sep, 3 (9): 721-32)

CSR is mediated through a recombination event between two switch (S) regions. S-regions are special DNA sequences located 5' of the constant region of antibodies. S-regions consist of highly repetitive G-rich sequences of 1-12 kilobases length. To initiate CSR, the S-region is transcribed from the cytokine controlled I exon promotor located upstream of the S-region. The transcript (sterile transcript/germ-line transcript) includes the S-region and the corresponding immunoglobulin constant region. It is spliced by fusing the I exon to the constant region exon deleting the S-region sequence. Sterile transcripts are not translated into protein. Association of sterile transcript with DNA forming RNA-DNA hybrids generates a single stranded R-loop structure that is accessible to the CSR recombinase AICDA[114, 116, 117].

The enzyme AICDA introduces deoxyuridine in the S-region of the DNA by deamination of cytidine[118]. The DNA might be processed by several DNA repair pathways leading to a DNA double strand break. CSR is completed after joining of two broken S-regions, generally the S-region of Cμ with one of the other constant regions by non-homologous end-joining proteins[114, 116].

Regulation of CSR. For induction of immunoglobulin class switching, two signals are required. The CSR activation signal occurs either depending on or independently of T helper cells. A second signal is provided by cytokines determining the isotype of the antibody.

The CSR induction signal provided by T helper cells is mediated by CD40 ligand interacting with CD40 receptor on the B cell. Furthermore, T helper cells secrete cytokines affecting the immunoglobulin isotype. The Th-1 cytokine INF-γ leads to switching to IgG2a and IgG3. The Th-2 cytokine IL-4 mediates switching to IgE and IgG1.

T helper cell independent signals inducing immunoglobulin class switching are mediated by the B lymphocyte stimulator protein (BAFF or BLyS) and by a proliferation-inducing ligand (APRIL). BAFF is found in a membrane bound and in a soluble form, while APRIL only functions as soluble factor. BAFF is primarily expressed by cells of the innate immunity such as neutrophils, but also by monocytes, macrophages, DCs, and activated T cells. APRIL is expressed by monocytes, macrophages, DCs, and T cells. BAFF and APRIL are structurally related and both are members of the TNF family. They are induced through LPS, CD40 ligand, and cytokines like IL-10, IFN-γ, and IFN-α. BAFF binds to three receptors selectively expressed by B cells, including transmembrane activator and calcium modulator and cyclophylin ligand interactor (TACI), B cell maturation antigen (BCMA), and BAFF receptor (BAFF-R or BR3). APRIL only interacts with BCMA and TACI, but not with BAFF-R. Binding of receptors by BAFF or APRIL activates a CD40-like pathway enhancing B cell

survival, maturation, and immunoglobulin class switching to IgG, IgA, and IgE in presences of cytokines like IL-4, IL-10, and TGF-β[119-121]. Over-expression of BAFF in mice not only augments B cell proliferation, but also T helper cell expansion. Furthermore, over-expression of BAFF leads to an induction of Th-1 response and enhanced incidence of autoimmune diseases[122].

CSR to IgE. Class switching to IgE is under tight control. The principal regulation event is induction of the Cε germ-line transcription. The Th-2 cytokine IL-4 and to a minor extend IL-13 induces Cε germ-line transcription through activation of the transcription factor STAT6 (signal transducer and activator of transcription 6). IL-18 also promotes class switching to IgE in an indirect manner. IL-18 enhances CD40 ligand expression on T helper cells and the release of IL-4, IL-10, IL-13, and IFN-γ[123].

IFN-γ inhibits the development of Th-2 cells and therefore the release of IL-4 and IL-13. Additionally, it blocks Cε germ-line transcription through direct interaction with B cells. IL-21 represses IgE production by inhibiting Cε germ-line transcription via unkown mechanisms[124, 125]. The B cell surface receptors CD45, cytotoxic T lymphocyte antigen (CTLA) 4, low affinity IgE receptor CD23, and the B cell receptor seem to inhibit Cε germ-line transcription, too. B cell lymphoma 6 (BCL6) inhibits Cε germ-line transcription by binding to the STAT6 sites on the Iε promotor. Furthermore, mice lacking BCL6 over-express Th-2 cytokines and have increased eosinophilia. TGF-β induces the expression of inhibitor of DNA binding (ID) 2. ID2 has a dual effect on IgE CSR. First, it regulates Th-1/Th-2 balance by regulating DC and secondly, it suppresses the Cε germ-line transcription in B cells[123, 126, 127]. We found that only T helper cell dependent mechanisms are responsible for CSR to IgE in contrast to T helper cell independent mechanisms (Chapter 2).

Allergy. Allergens are a certain type of antigens that induce IgE antibodies. Plasma cells of patients suffering from allergies and asthma secrete IgE instead of IgG in response to allergens and have therefore elevated serum levels of IgE. The predisposition to produce IgE antibodies is called atopy. IgE binds to Fc ε receptors on mast cells and basophils (Figure 6). These cells have granules containing pharmacologically active mediators. Basophils circulate in the blood stream making out about 0.5-1.0% of blood cells. Mast cells are found in tissues like skin or mucous membrane surfaces of the respiratory and gastrointestinal tracts. The skin contains around 10'000 mast cells per mm^3. Cross-linking of IgE bound to Fc ε receptors by allergens leads to degranulation of the mast cells and basophils. Inflammatory mediators like histamine, leukotrienes, prostaglandins, and cytokines like IL-4, IL-5, IL-6, and TNF are released. The mediators cause smooth-muscle contraction, increased vascular permeability, and vasodilatation resulting in the allergic symptoms.

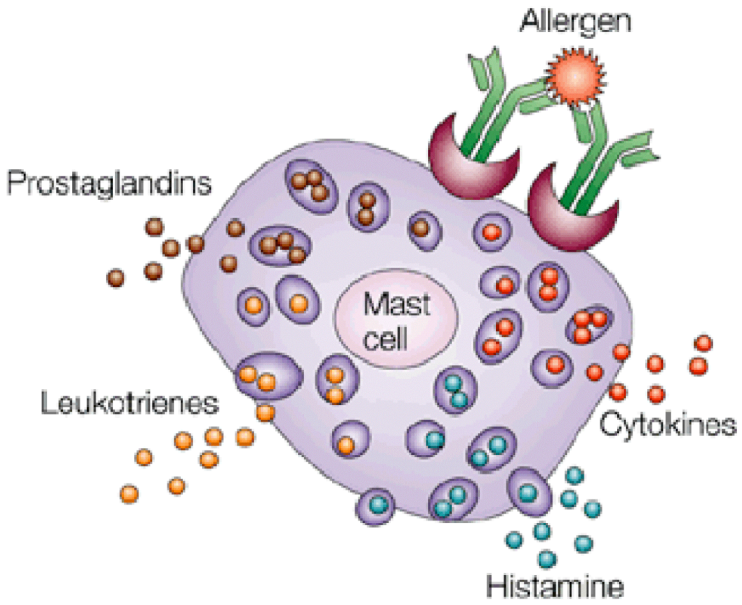

FIGURE 6. Mast cell and basophil degranulation. Cross-linking of IgE bound to Fc ε receptors on mast cells and basophils leads to secretion of inflammatory mediators like histamines, leukotrienes, prostaglandins, and cytokines resulting in allergic symptoms. (Adapted from Geha et al., Nat Rev Immunol. 2003 Sep, 3 (9): 721-32)

5. Objectives of this study

For a long time, the development of allergic diseases has been thought to be largely based on genetic predisposition. More recent epidemiological studies have shown that environmental factors are important, too. Not all children with a genetic predisposition develop allergic diseases. The hygiene hypothesis proposes that living in 'dirty' environments, as we find it on a farm, protects many of these children against the development of allergic disorders.

The immunological mechanisms underlying the hygiene hypothesis are the principal issue of this study. The prevailing immunological interpretation of the hygiene hypothesis is that decreased exposure to environmental microbial components or infections result in a shift of the Th-1/Th-2 balance. Reduced cellular activation through TLR by microbes in the environment reduces IL-12 and INF-γ secretion of APC resulting in development of Th-2 responses, finally resulting in allergic diseases. This interpretation of the hygiene hypothesis seems to be too simple. First, not only Th-2 associated diseases have increased over the past decades in parallel with improved hygiene but also Th-1 associated inflammatory and autoimmune diseases[4, 128]. Secondly, infection with helminth parasites induces a strong Th-2 response with eosinophilia, mucosal mastocytosis, and production of IgE. Children having parasitic infection suffer less from allergies than uninfected[129, 130].

Recently, Treg cells have been proposed to serve as immunological explanation for the hygiene hypothesis due to their suppressive activity on Th-1 and Th-2 effector cells. Furthermore, Treg cells are induced during allergen injection immunotherapy leading to suppression of Th-2 responses and to a switch from IgE to IgG4 antibody production[131]. But the findings that Treg cells are inactivated after TLR stimulation with bacterial components is in contrast to the protective role of bacterial components.

In chapter 2 of this study we checked whether these proposed immunological interpretations of the hygiene hypothesis were reproducible in the children of the ALEX and the PARSIFAL studies. We investigated whether the protective effect of environmental exposure of children to microbes was due to a shift in Th-1/Th-2 differentiation or due to altered generation of Treg cells. Therefore, we measured the expression of marker genes of T helper cells in blood leukocytes from children and assessed correlations between their expressions and exposure to environmental factors such as endotoxin in mattresses and living rooms, stable and animal contact of the mother during pregnancy, or living on a farm.

Since the innate immune system not only directs T helper cell differentiation but also inflammatory responses and T helper cell independent immunoglobulin isotype switching, we investigated whether regulatory molecules of the TLR signaling cascade have a protective role against allergies and inflammation in children. The expression of the SOCS-1 gene and of the ST2 gene has been described to be regulated by TLR ligands, thereby inducing endotoxin tolerance. This, in turn, was associated with protection against inflammatory and autoimmune diseases for SOCS-1 and with protection against allergies for ST2. We measured the expression of these genes in blood leukocytes of the children of the ALEX and PARSIFAL studies and assessed correlations with exposure to microbial components in the environment and with markers of inflammatory and allergic responses (Chapter 3 and 4).

Next, we investigated whether MHC class II molecules, additionally to their role in initiating adaptive immune responses, play a role in the regulation of the innate immune system, too. We studied this with cell culture experiments, where we co-expressed HLA-DR1 and TLR2 and confirmed our results in a mouse model (Chapter 5).

In the PARSIFAL cohort, we had a substantial number of women who stopped working on the farm after pregnancy, enabling us to distinguish between prenatal and early childhood exposure. Epidemiological analysis showed that prenatal exposure of the children to stable and farm animals was highly protective against the development of allergies. We thus were interested in possible changes in innate immunity. We investigated this in two ways. First, we studied whether expression of CD14, TLR2, and TLR4 correlated with prenatal exposure of the PARSIFAL children. We chose these three receptors because their expression was strongly regulated through farm-lifestyle (Chapter 6)[27]. In a second part, we studied whether a

single bacterial challenge was enough to induce persistent changes in the innate immune system indicating a memory function. To assess this, we designed a mouse model where we treated newborn mice with bacterial components. After three months, we assessed the expression of different genes of innate immunity in organs of the immune system (Chapter 7).

In this work, we found that the proposed mechanisms underlying the hygiene hypothesis have no relevance in protecting farm children against allergies. Rather, we observed that regulatory mechanisms of the innate immune system are able to reduce inflammatory responses and IgE levels of children.

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CHAPTER 2

Markers of the innate immune system but not of T helper cell phenotypes are associated with protection of farmers' children against allergies

Written as manuscript for publication

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Abstract

Background: A shift of T helper cell balance towards T helper cell type (Th) 1 or generation of T regulatory (Treg) cells has been proposed as immunological basis of the hygiene hypothesis. Objective: We investigated whether a skewing of the T helper cell balance or a change in the generation of Treg cells could be observed in farmers' children. Furthermore, we assessed if either T cell dependent or T cell independent mechanisms resulted in immunoglobulin isotype switching to IgE. Methods: The expression of T helper cell makers and Cε germ-line transcripts was assessed in blood leukocytes of farmers' and non-farmers' children from the Swiss subsample of the PARSIFAL population using quantitative real-time PCR. Results: We found that the expression of some genes associated with Th-1 differentiation correlated with environmental conditions created by farming. However, as indicated by the expression of transcription factors T-bet, GATA-3, and FOXP3, protection against allergies in the farming population investigated here could not solely be explained by a skewing of Th-1/Th-2 balance or a change in the generation of Treg cells. Furthermore, T cell activation assessed by the expression of CD40 ligand, T-bet, GATA-3, and FOXP3 correlated with immunoglobulin isotype switching to IgE. In contrast, gene expression of pattern recognition receptors regulated via the exposure to environmental factors and the expression of BAFF was negatively associated with immunoglobulin isotype switching to IgE. Conclusion: Mechanisms of the innate immune system may constitute the immunological basis of the hygiene hypothesis.

Introduction

The hygiene hypothesis was proposed after observing a lower risk of hay fever and atopic sensitization in children with more siblings[1]. The protective effect was attributed to elevated infections during childhood. Other findings have supported this hypothesis. Early enrollment in a day nursery, for example, has protective effects against the development of allergies while Italian military students with antibodies against hepatitis A virus have a lower prevalence of atopy and atopic respiratory diseases[2, 3].

Other studies have shown that a rural lifestyle, in addition to infections, protect children against allergies[4]. Contact to farm animals in particular was demonstrated to be highly protective[5, 6]. The protective effects of a farm were attributed to exposure to higher loads of bacterial components as shown for endotoxin, a lipopolysaccharide (LPS) of the outer layer of the cell membrane of Gram-negative bacteria[7, 8]. Furthermore, child's consumption of non-pasteurized milk and working of the mother in stable or with stable animals during pregnancy have protective effects on children[9]. Farmers' children have reduced IgE levels in serum and

their blood leukocytes express more Toll-like receptors (TLR), the principal family of pattern recognition receptors (PRR) of the innate immune system[7, 9-11].

The leading immunological interpretation of the hygiene hypothesis is that decreased infections or exposure to environmental microbial components result in a shift of T helper cell type (Th)-1/Th-2 balance[1, 12, 13]. Cytokines and co-stimulatory molecules of innate immunity direct T helper cell activation and differentiation. The Th-1 response is initiated by interleukin (IL)-12 and is characterized by IL-2, tumor necrosis factor (TNF)-β, and gamma interferon (IFN-γ) secretion. IFN-γ inhibits Th-2 cell differentiation and mediates secretion of IgG antibodies by B cells. Th-1 differentiation is controlled by transcription factor T-bet and is associated with inflammation and autoimmunity[14]. IL-4 induces a Th-2 response. Th-2 specific genes are regulated by transcription factor GATA-3 enhancing IL-4, IL-5, and IL-13 expression. These cytokines inhibit Th-1 differentiation and induce immunoglobulin class-switch recombination (CSR) to Cε in B cells. Thereby, Th-2 response is associated with allergic diseases[15]. The binding of ligands to a TLR leads to the translocation of NF-κB to the nucleus and to MAP kinase activation ending in the secretion of inflammatory, Th-1 associated cytokines: TNF and IL-12[16, 17]. The absence of TLR activating signal caused by fewer infections or low levels of microbes in environment decreases IL-12 secretion of the innate immune system resulting in development of Th-2 responses mediating immunoglobulin CSR to Cε in B cells[16].

Recently, T regulatory cells (Treg) were proposed as another immunological explanation for the hygiene hypothesis due to their suppressive activity on Th-1 and Th-2 effector cells[18]. Enhanced hygiene conditions may lead to loss of suppressive activity of Treg cells[19]. The Treg cell phenotype is controlled by transcription factor FOXP3. The suppressive activity depends on cell to cell contact[20] in addition to the secretion of immunosuppressive cytokines IL-10 and TGF-β[21, 22].

Immunoglobulin CSR exchanges the constant region of antibody, generally Cμ (IgM) to Cγ (IgG), Cα (IgA), or Cε (IgE). Immunoglobulin CSR is induced by T helper cells through the interaction of CD40 ligand (CD40L) with CD40 on B cells. Released cytokines determine the isotype to which the antibody constant region switches. The innate immune system has the ability to induce immunoglobulin CSR, too, without the contribution of T helper cells. T helper cell independent immunoglobulin CSR is mediated by various molecules such us BAFF (B lymphocyte stimulator protein) or APRIL (a proliferation-inducing ligand), two soluble factors mainly expressed by cells of the innate immune system. BAFF binds to three receptors primarily expressed by B cells, including transmembrane activator and calcium modulator and cyclophylin ligand interactor (TACI), B cell maturation antigen (BCMA), and BAFF receptor (BAFF-R or BR3). APRIL only interacts with BCMA and TACI, but not with

BAFF-R. The binding of BAFF or APRIL by receptors enhances B cell survival, maturation, proliferation and immunoglobulin CSR[23-25].

CSR is mediated by a recombination event between two switch (S) regions. S-regions are special DNA sequences located 5' of the constant region of antibodies consisting of highly repetitive G-rich sequences of 1-12 kb in length. To initiate CSR, the S-region is transcribed from the cytokine controlled I exon promotor located upstream of the S-region. The transcripts (sterile transcripts/germ-line transcripts, GLT) include the S-region and the corresponding immunoglobulin constant region. They are spliced by fusing the I exon to the constant region exon and deleting the S-region sequence. GLT are not translated into protein. Association of a GLT with the DNA forming RNA-DNA hybrids generates a single stranded R-loop structure that is accessible for the CSR recombinase AICDA (activation-induced cytidine deaminase)[26-28]. AICDA introduces deoxyuridine in the S-region of the DNA by deamination of cytidine[29]. The DNA might be processed by several DNA repair pathways leading to a double strand break in the DNA. CSR is completed after joining of two broken S-regions by non-homologous end-joining proteins[26, 27].

The goal of the PARSIFAL (Prevention of Allergy Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle)[30] study was to identify factors making rural lifestyle so protective against allergies in children[9]. Here, we investigated whether the proposed immunological mechanisms for the hygiene hypothesis were relevant in a human system in a Swiss subsample of the PARSIFAL study. We assessed whether factors previously identified as highly protective against the development of allergic diseases shifted T helper cell differentiation towards Th-1 or enhanced the generation of Treg cells, thereby leading to less immunoglobulin CSR to IgE in these children. We found that none of the protective environmental factors was associated with a shift towards Th-1 or Treg cell differentiation. Furthermore, we found that the expression of the CD40L gene as well as that of T helper cell marker genes were positively associated with immunoglobulin CSR to IgE. By contrast, the expression of both, the BAFF gene and pattern recognition genes of the innate immune system, correlated with lower immunoglobulin CSR to IgE. These results indicate that exposure to microbial components, as they are found on a farm, activates mechanisms of the innate immune system leading to protection against immunoglobulin CSR to IgE and thereby protecting against the development of allergies, while T cell response remains unaffected.

Methods

Population and questionnaire. We assessed the expression of relevant T helper cell marker genes and of genes of the innate immune system in the Swiss branch of the

PARSIFAL study. RNA samples were collected from 195 farm and 127 reference children (95.3% of children who provided blood samples) to analyze gene expression[9]. Parents of participating children were invited to fill out questionnaire used in this study. Questions on farming lifestyle and farm exposures were derived from the internationally validated International Study of Asthma and Allergies in Childhood II[31] questionnaire and the Allergy and Endotoxin study[32], respectively.

Measurement of endotoxin and fungal extracellular polysaccharides in dust samples. Endotoxin and fungal extracellular polysaccharides (EPS) were measured in mattress dust samples of 83.9% of children with complete gene expression data (n = 270). Sampling and detection methods are described elsewhere[33]. In brief, endotoxin was measured with the kinetic chromogenic Limulus Amebocyte Lysate test (Bio Whittaker) and EPS with a specific sandwich enzyme immunoassay for EPS of *Aspergillus* and *Penicillium* species[9, 34]. We divided the children in three groups due to the amount of endotoxin or EPS in mattress' dust samples. The expression values of genes of the group with highest endotoxin or EPS amounts were related to group with the lowest.

RT-PCR and quantitative real-time PCR (TaqMan®). The total RNA was isolated as described [9] and stored at minus 80°C. For reverse transcription (RT) of RNA we used 300ng of total RNA in a final volume of 30µl and added adequate amounts of TaqMan® Reverse Transcription Reagents (Applied Biosystems). The quantification of Cε germ-line transcripts we used the ABI Prim 7700 Sequence Detection System (Applied Biosystems) and the following primers: forward 5'-ACAGGCACCAAATGGACGAC-3', reverse 5'-TTGCAGCAGCGGGTCAA-3'. The minor groove binding probe had the sequence 5'-CACAGAGCCCATCCG-3'. The quantification of the other genes was performed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems) using the TaqMan® low density array (LDA) system of Applied Biosystems. The determined gene expression values were normalized to the parallel measured endogenous controls 18S rRNA. We analyzed the data with the comparative Ct method according to the manufacturer's instructions (Applied Biosystem).

Results

We investigated whether living on a farm or exposure to environmental factors associated with protection against the development of allergies resulted in skewing of Th-1/Th-2 balance or in induction of Treg cells in children of a Swiss subsample of the PARSIFAL study. We measured the expression of marker genes of Th-1, Th-2, and T reg cells in blood leukocytes of children by quantitative real-time PCR and related it to environmental exposure factors (Table 1). The expression of T-bet gene, being the most specific marker for Th-1, failed to

associate with environmental exposure factors. Furthermore, we assessed expression of chemokine receptors CCR5 and CXCR3 as Th-1 markers[35, 36]. Only CXCR3, which is also expressed on mast cells[37], showed a positive correlation with analyzed environmental exposure factors. Expression of Th-2 marker genes, transcription factor GATA-3 and chemokine receptors CCR4 and CCR8[35, 36] were positive associated with environmental factors such as farming, exposure of the mother during pregnancy, child's consumption of non-pasteurized milk, or fungal extracellular polysaccaraides (EPS) in child's mattress. By contrast, expression of the Th-2 associated chemokine receptor CCR3 gene was significantly decreased. CCR3 is not selectively expressed on Th-2 cells, but is also found on eosinophiles and basophiles[38]. Expression of Treg cell marker gene FOXP3 was unaffected by environmental exposure factors.

TABLE 1. **Association between environmental exposure factors and T helper cell marker**

Environmental exposure factor	Th-1 associated			Treg associated
	T-bet	CCR5	CXCR3	FOXP3
Being a farm child	1.03 (0.87-1.23)	1 (0.77-1.28)	2.02 (1.5-2.7)**	0.97 (0.82-1.15)
Number of animals the mother had contact during pregnancy	0.97 (0.92-1.03)	1 (0.93-1.09)	1.23 (1.12-1.35)**	1 (0.95-1.05)
Maternal work in stable during pregnancy	1.04 (0.87-1.24)	0.91 (0.7-1.18)	1.82 (1.35-2.46)**	1.01 (0.85-1.19)
Child's consumption of non-pasteurized milk	0.99 (0.82-1.18)	1.03 (0.79-1.33)	1.74 (1.29-2.35)**	0.94 (0.79-1.11)
Endotoxin in mattress (EU/g dust)	0.89 (0.72-1.1)	1.06 (0.78-1.45)	1.39 (0.96-2)+	1.08 (0.88-1.32)
EPS in mattress (EPS/g dust)	0.99 (0.8-1.23)	0.98 (0.72-1.33)	1.51 (1.05-2.17)*	1.05 (0.86-1.28)
Environmental exposure factor	Th-2 associated			
	GATA-3	CCR3	CCR4	CCR8
Being a farm child	1.25 (1.04-1.5)*	0.67 (0.51-0.9)**	1.22 (0.97-1.54)+	2.24 (1.39-3.6))**
Number of animals the mother had contact during pregnancy	1.05 (0.99-1.11)	0.9 (0.83-0.99)*	1.09 (1.01-1.17)*	1.23 (1.07-1.43)**
Maternal work in stable during pregnancy	1.25 (1.03-1.5)*	0.77 (0.58-1.04)+	1.27 (1-1.61)*	2.66 (1.64-4.31)**
Child's consumption of non-pasteurized milk	1.21 (1-1.46)*	0.7 (0.53-0.93)*	1.27 (1.01-1.6)*	2.11 (1.3-3.42)**
Endotoxin in mattress (EU/g dust)	1.07 (0.85-1.34)	0.88 (0.62-1.25)	1.29 (0.97-1.71)+	1.47 (0.83-2.58)
EPS in mattress (EPS/g dust)	1.27 (1.02-1.58)*	0.95 (0.67-1.34)	1.09 (0.82-1.44)	1.39 (0.79-2.43)

All values adjusted for sex, age, and parental history of atopy. Measure of effect size for all values: geometric means ratios and 95% confidence intervals; except for CCR8: Odds ratio and 95% confidence intervals

+: $0.1 > p > 0.05$, *: $0.05 > p > 0.01$, **: $p < 0.01$

T helper cells exhibit their function via the release of a specific cytokine pattern. We investigated whether environmental exposure factors correlated with cytokine pattern secreted by T helper cells (Table 2). Gene expression of Th-1 differentiation inducing cytokine IL-12 was not affected by environmental exposure factors. But gene expression of IFN-γ, secreted by Th-1 cells was clearly down-regulated in blood leukocytes of children living in farming lifestyle. Gene expression of Th-2 associated cytokine IL-4 was enhanced in children living on a farm being in line with induced Th-2 transcription factor GATA-3. IL-13 gene expression was unaffected by the environmental exposure factors. The expression of the inhibitory cytokine IL-10 was highly induced while the expression of TGF-β was only slightly increased related to environmental exposure factors. Therefore, farming lifestyle failed to skew T helper cell balance or induced Treg cell differentiation, but decreased INF-γ and enhanced IL-10 and TGF-β secretion.

TABLE 2. **Association between environmental exposure factors and cytokines**

Environmental exposure factor	Cytokine					
	IL-12α	IFN-γ	IL-4	IL-13	IL-10	TGF-β
Being a farm child	0.94 (0.73-1.21)	0.45 (0.32-0.63)**	1.73 (1.08-2.77)*	0.69 (0.3-1.59)	2.43 (1.7-3.48)**	1.11 (0.98-1.25)+
Number of animals the mother had contact during pregnancy	0.97 (0.9-1.05)	0.78 (0.7-0.86)**	1.08 (0.93-1.24)	0.91 (0.72-1.16)	1.32 (1.18-1.48)**	1.05 (1.01-1.09)**
Maternal work in stable during pregnancy	1.11 (0.86-1.43)	0.49 (0.35-0.7)**	1.56 (0.98-2.51)+	0.58 (0.25-1.33)	2.28 (1.58-3.29)**	1.15 (1.02-1.3)*
Child's consumption of non-pasteurized milk	0.99 (0.77-1.28)	0.63 (0.45-0.89)**	1.58 (0.97-2.55)+	1.57 (0.68-3.61)	2.13 (1.48-3.07)**	1.16 (1.02-1.3)*
Endotoxin in mattress (EU/g dust)	1.05 (0.78-1.43)	0.78 (0.51-1.19)	0.96 (0.54-1.7)	0.93 (0.32-2.67)	2.06 (1.33-3.21)**	1.09 (0.94-1.26)
EPS in mattress (EPS/g dust)	1.27 (0.94-1.71)	0.61 (0.4-0.93)**	1.18 (0.68-2.07)	0.73 (0.27-1.97)	2.04 (1.32-3.16)**	1.11 (0.96-1.28)

All values adjusted for sex, age, and parental history of atopy. Measure of effect size for all values: geometric means ratios and 95% confidence intervals; except for IL-4 and IL-13: Odds ratios and 95% confidence intervals
+: 0.1 > p > 0.05, *: 0.05 > p > 0.01, **: p < 0.01

We were also interested whether T helper cell balance was responsible for immunoglobulin CSR to IgE in blood leukocytes of children. We measured the expression of Cε GLT by quantitative real-time PCR. Since immunoglobulin CSR primarily takes place in lymph nodes, we also determined the expression of AICDA and Bach2 genes, two molecules absolutely necessary for immunoglobulin CSR[39]. Expression of AICDA and Bach2 genes was positively associated with expression of Cε GLT (Geometric means ratio 1.49, $p<0.05$ for AICDA and 1.62, $p<0.01$ for Bach2) indicating that immunoglobulin CSR took place in blood leukocytes of children.

We then related expression of Cε GLT to gene expression of CD40L and to expression of T-bet, GATA-3, and FOXP3 (Figure 1). We found positive association between immunoglobulin CSR inducing molecule CD40L expressed on T cells and Cε GLT. Furthermore, expression of transcription factors T-bet, GATA-3, and FOXP3 correlated with expression of Cε GLT indicating that enhanced T cell activation associated with elevated immunoglobulin isotype switching to IgE.

Since TLR expression of children was controlled by environmental exposure factors[9, 10], we investigated whether mechanisms of the innate immune system may influence immunoglobulin CSR to IgE. We assessed gene expression of TLR2, TLR4, CD14, MD-2, and TREM1 and of immunoglobulin CSR inducing ligands APRIL and BAFF in blood leukocytes of children (Figure 1). We found that the expression of BAFF gene was negatively associated with expression of Cε GLT, while APRIL expression failed to correlate. Furthermore, gene expressions of pattern recognition receptors involved in recognition of bacterial components were negatively associated with expression of Cε GLT.

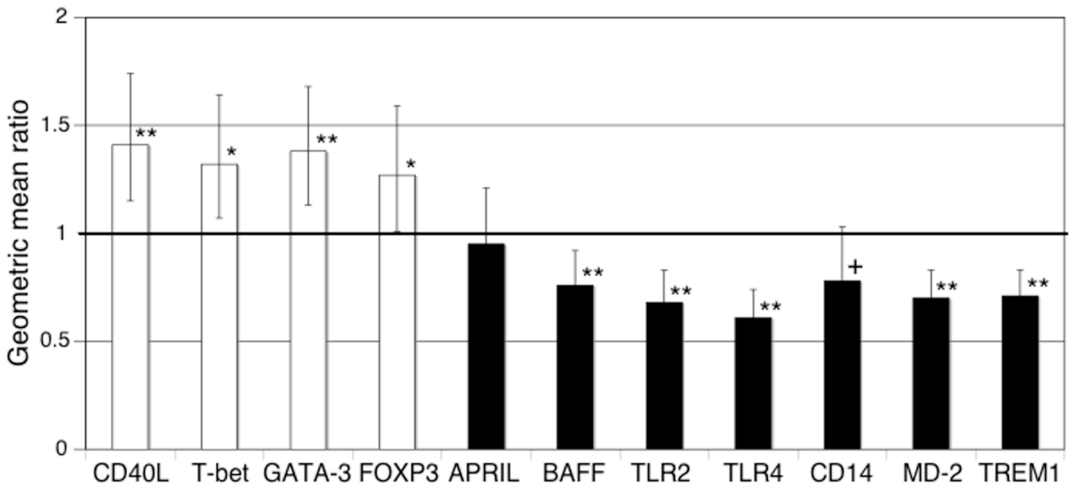

FIGURE 1. Expression of T cell and innate immunity marker genes related to expression of Cε GLT in blood leukocytes of children. We associated expression of CD40L gene and of T helper cell or Treg cell marker genes to expression of Cε GLT (white bars). Furthermore, expression of PRR and immunoglobulin CSR inducing ligands BAFF and APRIL genes were related to expression of Cε GLT (black bar). Measure of effect size: geometric means ratios and 95% confidence intervals; all estimates adjusted for farming status, sex, age, and parental history of atopy; $^{+}$: $0.1 > p > 0.05$, *: $0.05 > p > 0.01$, **: $p < 0.01$.

Finally, we were interested whether the expression of APRIL and BAFF gene associated either with environmental exposure factors or with TLR gene expression to assess a link between these ligands and the hygiene hypothesis. Expression of APRIL and BAFF gene failed to correlate with environmental exposure factors (data not shown), but we found positive associations between APRIL and BAFF gene expression and the expression of TLR genes (Table 3).

TABLE 3. **Association between gene expression of PRR and APRIL or BAFF**

Pattern recognition receptor		
	APRIL	BAFF
TLR2	1.43 (1.31-1.57)**	1.67 (1.52-1.84)**
TLR4	1.58 (1.45-1.71)**	1.85 (1.7-2.02)**
CD14	2.06 (1.87-2.27)**	1.76 (1.55-2.01)**
MD-2	1.53 (1.43-1.64)**	1.72 (1.59-1.85)**
TREM1	1.43 (1.34-1.53)**	1.41 (1.3-1.53)**

All values adjusted for farming status, sex, age, and parental history of atopy. Measure of effect size for all values: geometric means ratios and 95% confidence intervals

+: $0.1 > p > 0.05$, *: $0.05 > p > 0.01$, **: $p < 0.01$

Discussion

In this study, we investigated whether T cell dependent or innate immune mechanisms led to immunoglobulin isotype switching to IgE in context of exposure to environmental microbial components in children. Our data showed that the widely proposed shift in T helper

cell balance to enhanced Th-1 differentiation via exposure to microbes, as they are found in farming environments, failed to take place in children. It seemed that farmers' children had even more Th-2 cells as indicated by enhanced GATA-3, CCR4, CCR8 and IL-4 gene expression while IFN-γ gene expression was clearly reduced. Our findings coincide with other observations indicating that skewing Th-1/Th-2 balance may not be the immunological mechanism for the hygiene hypothesis. Not only have Th-2 associated diseases increased over the past decades in parallel with elevated hygiene conditions, but also Th-1 associated inflammatory and autoimmune diseases[18, 40, 41]. There are also patients with concurrent allergic diseases and autoimmunity[13]. And helminth parasite infection induces a strong Th-2 response with eosinophilia, mucosal mastocytosis, and increased production of IgE. Interestingly, children having parasites infection suffer less from allergies than uninfected classmates[42, 43]. Our results together with these additional observations make skewing of T helper cell differentiation implausible as immunological mechanism for the hygiene hypotheses.

Expression of Treg cells marker failed to correlate with environmental factors, too. Anyway, Treg cells as explanation for the hygiene hypothesis are unlikely because TLR-mediated IL-6 production has been shown to block the suppressive activity of Treg cells[44, 45]. Furthermore, stimulation of TLR2 or TLR8 bearing Treg cells by TLR ligands reverses their suppressive function[46, 47]. Farming lifestyle induced the expression of suppressive cytokine IL-10. IL-10 inhibited inflammatory cytokine production by innate immunity and elicited anergy in T cells. But its role in allergic diseases remains controversial. IL-10 produced by dendritic cells promoted the development of Th-2 cells, while IL-10 was increased in patients with allergic asthma. In contrast, IL-10 appeared to have a role in allergen-specific immunotherapy, too[19, 48, 49]. In our study, we failed to find association between IL-10 expression and allergic disorders or IgE in serum (data not shown). But the enhanced IL-10 secretion together with the reduced IFN-γ level in farmers' children indicate a diminished inflammatory responses and may thereby protect against the development against allergic diseases.

Immunoglobulin CSR to IgE in peripheral blood was enhanced via T helper cell activation. We found positive association between all phenotype-specific T helper cell markers and Cε GLT. By contrast, pattern recognition receptor gene expression and the expression of BAFF seemed to protect against immunoglobulin CSR to IgE. Our study failed to explain how the innate immune system constitutes this protection. Principally, BAFF enhances B cell proliferation and immunoglobulin isotype switching to all constant regions[23]. It is possible that BAFF directed immunoglobulin CSR preferable to switching to Cγ. Furthermore, BAFF suppresses Th-2 response[50-52]. This mechanism was rather unlikely, as we failed to observe a shift in T helper cell differentiation in farmers' children. Another role of BAFF is to elevate

TLR dependent B-1 cell activation[53]. B-1 cells are naturally occurring B cells secreting germ-line encoded IgM and IgG and contributing to the first line defence of the immune system. Enhanced activation of B-1 cells might have led to decreased B-2 response, thereby reducing the generation of IgE mediated by B-2 cell.

These data indicate that microbial level or other environmental exposure factors associated with reduced hygienic conditions of a farm failed to influence T cell differentiation. Skewing of the T helper cell balance may only become important if microbial levels are much higher, as it is the case during an infection. By contrast microbial levels of a farm left a mark on the innate immune system[9, 10]. Thereby induced mechanisms of the innate immune system reduce the inflammatory responses and have the potential to protect against allergies and may constitute the central immunological basis of the hygiene hypothesis.

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CHAPTER 3

Suppressor of cytokine signaling (SOCS)-1: a link between environmental exposure to microbial components and inflammatory responses and IgE

Written as manuscript for publication

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Introductory paragraph

Epidemiological studies have shown an increase in the incidence of allergic diseases in western countries during the past decades in parallel to a decrease in infectious diseases[1]. The hygiene hypothesis proposes that living in environments rich in microbes or predisposing to infections and thereby favoring T helper cell (Th) differentiation towards the Th-1 phenotype and away from the allergy promoting Th-2 phenotype underlies this protection against allergies[2-5]. However, similar environmental conditions have been found to protect against Th-1 associated autoimmune and auto-inflammatory diseases, too[6]. The frequency of these Th-1 associated diseases has risen in the past decades in western countries similar to Th-2 associated allergies[1, 7, 8]. This might be considered an immunological contradiction. Thus, we searched for immunological mechanisms independent of the Th-1/Th-2 balance.

Suppressor of cytokine signaling (SOCS)-1 protein is induced in response to microbes and suppresses inflammatory and autoimmune responses in mice[9-11]. We found that in children environmental exposure to the microbial component lipopolysaccaride (LPS/endotoxin) resulted in elevated expression of the SOCS-1 gene, which, in turn, correlated to decreased INF-γ responses and IgE levels. Thus, SOCS-1 may be part of an anti-inflammatory feedback mechanism contributing to protection against both allergic and autoimmune diseases by environmental microbes.

Results and Discussion

The relationship between environmental microbial exposure and allergies in children has been investigated in a cross-sectional epidemiologic study (Allergy and Endotoxin, ALEX)[12-14]. We measured the expression of the SOCS-1 gene by quantitative real-time PCR in a subsample (n=43) of the ALEX cohort and determined the level of endotoxin in dust samples collected from the environment of the children. The exposure to endotoxin of the children ranged from 1,320 to 167,185 EU/m^2 (geometric mean 14,797 EU/m^2) in living rooms and from 2497 to 87,726 EU/m^2 (geometric mean 10,885 EU/m^2) in mattresses. The SOCS-1 gene expression in children's PBL was positively associated with the endotoxin level in environment (Figure 1). The correlation coefficient of the expression of the SOCS-1 gene with the endotoxin load in children's living room and mattress was 0.35 (p=0.0224) and 0.345 (p=0.0236), respectively. Multivariate linear regression showed that a tenfold increase in endotoxin load in living room was associated with a 1.57 fold increase in SOCS-1 gene expression in children's PBL (p=0.0086). This finding is in agreement with previously described results from mouse models[15-18], where SOCS-1 protein was up-regulated by Toll-like receptor (TLR) agonists such as LPS to negatively regulate LPS signaling. Macrophages

from SOCS-1 knock-out mice were hyper-responsive to LPS and produced increased amounts of pro-inflammatory cytokines[9]. SOCS-1 suppressed LPS induced NF-κB activation through direct interaction with interleukin-1 receptor-associated kinase (IRAK) and through degradation of the MAL (MyD88-adaptor like, also called TIRAP) protein, a cytosolic Toll-interleukin 1 receptor (TIR) domain containing adaptor protein[19, 20]. Thus, SOCS-1 is an important molecule contributing to LPS-tolerance.

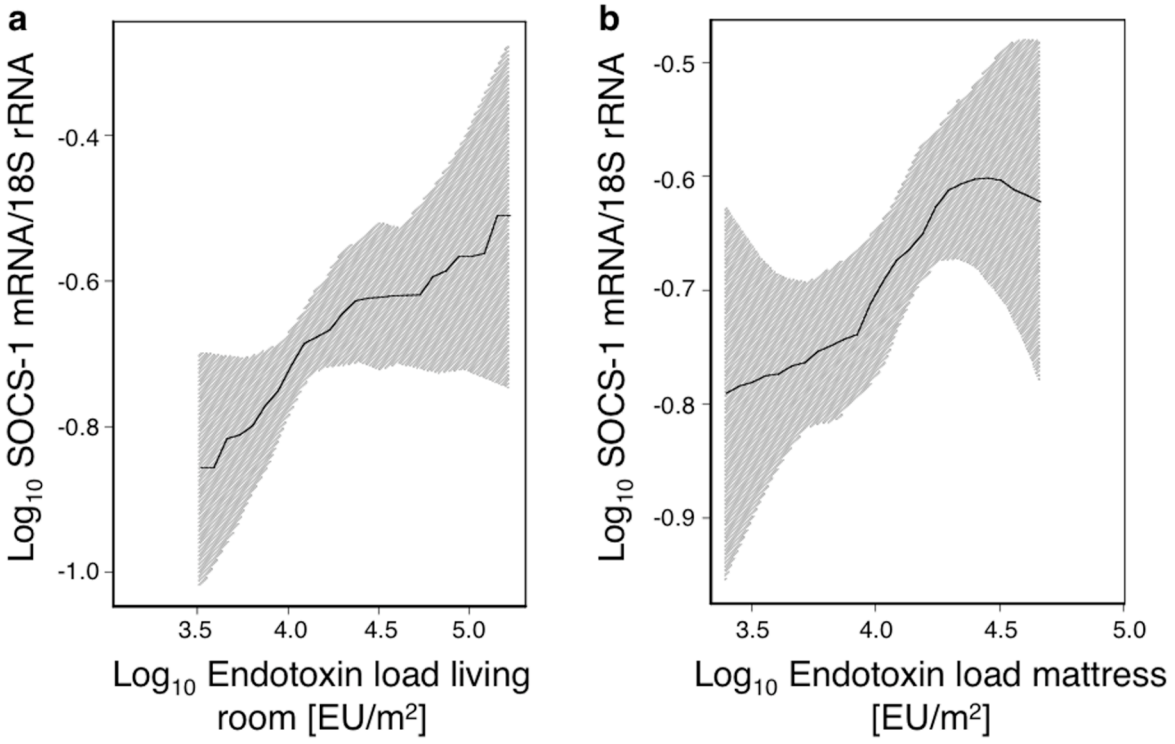

FIGURE 1. Smoothed plots of expression of the SOCS-1 gene in relation to endotoxin in children's environment. We related the SOCS-1 gene expression to endotoxin loads in the living room (panel a) and mattress (panel b) of children. All values (n=43) were log-transformed and adjusted for age and farming. A smoothing span of 0.8 was used for both graphs. The shaded area represents the 95% confidence interval.

Immunological investigations realized in the context of epidemiological studies showed that PBL of children living in an environment with elevated amounts of endotoxin secreted less inflammatory cytokines upon *in vitro* re-stimulation with LPS[12]. We therefore investigated whether SOCS-1 levels increased by the exposure to environmental endotoxin were sufficient to decrease the responsiveness of PBL to LPS. To assess a possible correlation between SOCS-1 expression and LPS-responsiveness *in vivo*, we stimulated PBL of children with LPS *in vitro* and related the amounts of secreted cytokines to the expression of the SOCS-1 gene (Figure 2). The SOCS-1 expression in PBL was inversely related to INF-γ release after LPS treatment (correlation coefficient of -0.32, p=0.0415). A tenfold increase in expression of the SOCS-1 gene was associated with a 9.9 fold decrease in the concentration of

INF-γ (p=0.0369). We observed no statistically significant relationships between SOCS-1 expression and the secretion of IL-5 and IL-10 (correlation coefficient of -0.07 (p=0.7348) for IL-5 and of -0.28 (p=0.7348) for IL-10). Thus, PBL of children living in an environment with a higher endotoxin load express higher levels of the SOCS-1 gene. This, in turn, is associated with decreased production of INF-γ upon stimulation with LPS *in vitro*. This can be interpreted as LPS-tolerance.

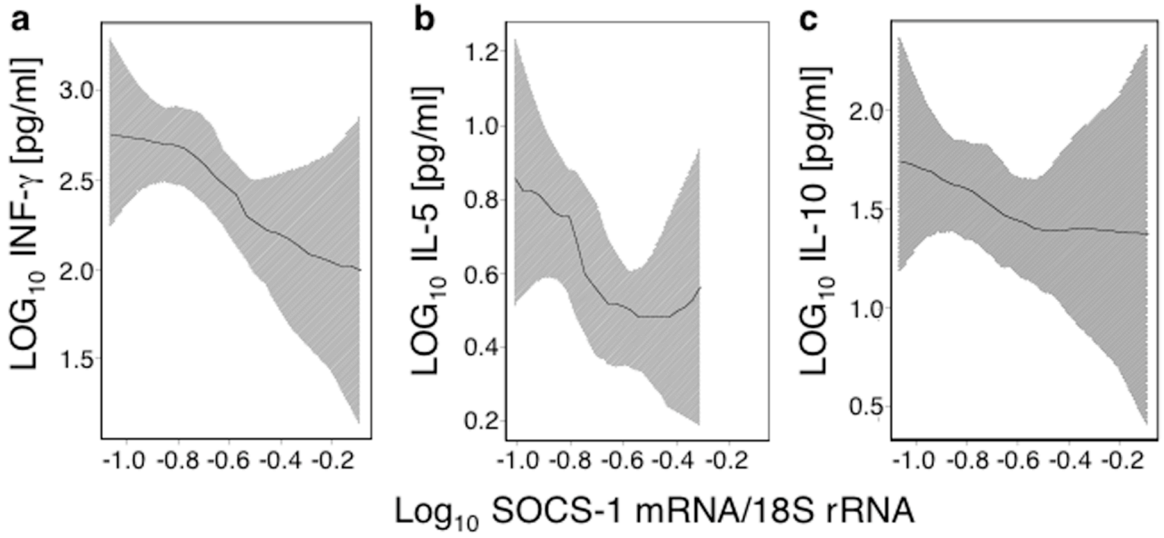

FIGURE 2. Smoothed plots of cytokines secreted upon LPS treatment of whole blood samples in relation to the expression of the SOCS-1 gene. We related INF-γ (panel a), IL-5 (panel b), and IL-10 (panel c) levels in supernatants following LPS stimulation of blood samples to expression of the SOCS-1 gene. All values (n=43) were log-transformed and adjusted for age and farming. A smoothing span of 0.75 was used for all three graphs. The shaded area represents the 95% confidence interval.

SOCS-1 knock-out mice had elevated serum IgE levels[10]. We thus investigated whether such a relationship was present in the children of the ALEX study, too. Despite the low numbers of samples we could analyze in this study, a trend towards an inverse correlation between the expression of the SOCS-1 gene and the IgE level could be observed, although not reaching statistical significance. A tenfold increase in the SOCS-1 gene expression was associated with a decrease in IgE level by a factor of 3.57 (p=0.1968) (Figure 3).

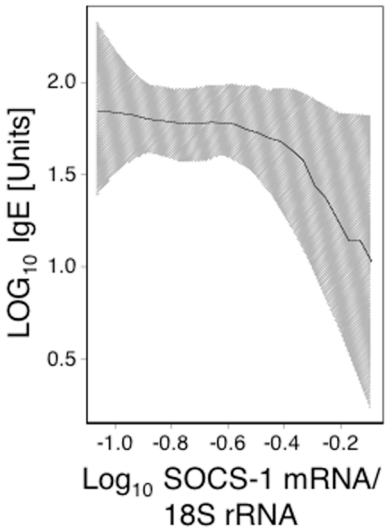

FIGURE 3. Smoothed plot of IgE levels in serum of children in relation to the expression of the SOCS1 gene. We related IgE levels in untreated serums of children to the expression of the SOCS1 gene. All values (n=43) were log-transformed and adjusted for age, farming, and family history of asthma or hay fever. A smoothing span of 0.8 was used for the graph. The shaded area represents the 95% confidence interval.

In summary, the microbial load in the environment correlates with elevated expression of the SOCS-1 gene in children, which, in turn, correlates with lower IgE levels and reduced inflammatory responses as shown by decreased levels of INF-γ. We propose that exposure to microbes induces inhibitory feedback mechanisms, such as up-regulation of the SOCS-1 protein. Such regulatory mechanisms of the innate immune system may contribute to the protection conferred by exposure to environmental microbes against both Th-2 and Th-1 associated diseases, i.e. allergic as well as autoimmune disorders.

Methods

Sample Collection. The ALEX study was a cross-sectional survey investigating the incidence of asthma and allergies in children from rural areas of Austria, Germany, and Switzerland. In a subgroup of the Swiss sample (n=43 children), we measured the expression of the SOCS-1 gene in peripheral blood leukocytes (PBL), assessed the cytokine production by whole blood after *ex vivo* LPS treatment, and determined the IgE level in serum.

Measurement of endotoxin (LPS) levels. We collected dust samples in participants' living rooms and mattresses and measured LPS contents using a kinetic limulus assay as previously described[12]. We expressed the amount of LPS in endotoxin units (EU) per square meter and designated it as endotoxin load.

IgE measurements and cytokine production of blood cells. We stimulated heparinized whole blood of children with LPS (10µg/ml) for 24h. Cytokine production and levels of IgE were assessed as previously described[12].

SOCS-1 gene expression. We assessed gene expression *ex vivo* with quantitative real-time PCR (TaqMan, Applied Biosytems), without further stimulation of the samples *in vitro*. The total RNA was extracted as described from PBL[13]. For reverse transcription (RT) we used 300ng of total RNA in a final volume of 30µl and the TaqMan reverse transcription reagents of Applied Biosystems. We performed quantitative real-time PCR with 3µl of RT solution in a final volume of 25µl and analyzed it with an ABI Prism 7700 Sequence Detection System™ (Applied Biosystems). To each reaction we added primers, probe, and 12.5µl TaqMan Universal PCR Master Mix™ (Applied Biosystems). The primers and probes were designed with the primer design software Primer Express™ (Applied Biosystem). To measure the SOCS-1 gene expression we used the following primer pair: 5'-GCGGAACTGCTTTTTCGC-3' (200nM) and 5'-ACGCGGATGCTCGTGG-3' (200nM), and a probe with the sequence 5'-CTTAGCGTGAAGATGGCCTCGGGA-3' (250nM). We related the expression of SOCS-1 gene to the expression of the endogenous control 18S rRNA determined using the following primer pair: 5'-AGTCCCTGCCCTTTGTACACA-3' (200nM) and 5'-GATCCGAGGGCCTCACTAAAC-3' (200nM), and a probe with the sequence 5'-CGCCCGTCGCTACTACCGATTGG-3' (250nM). We analyzed the results using the standard curve method as described[21]. For every primer and probe set, we performed control experiments assessing background signals by running the reaction without cDNA („no template controls“). We did not observe amplification in any of these “no template controls”, indicating that there was neither contamination nor unspecific fluorescence.

Statistical Analysis. We calculated Spearman’s correlation coefficients to examine the relationship between expression of the SOCS-1 gene and the level of endotoxin in the children’s environment, the amount of cytokines secreted after *ex vivo* stimulation of whole blood with LPS, and the levels of IgE. We log10 transformed all values. We applied linear regression models to determine the above relationships adjusted for effect of personal risk factors such as sex, age, family history of asthma or hay fever, allergic sensitization and farming (SAS 8.2 software). To visualize these associations, SOCS-1 gene expression predicted value resulting from a generalized additive regression model using local nonparametric smoothing with control for the mentioned covariates was plotted versus exposure to environmental endotoxin (S-Plus software). Similarly, we plotted the predicted values of cytokine concentrations and IgE levels versus expression of the SOCS-1 gene. The final models included farming and statistical significant covariates.

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CHAPTER 4

Exposure to environmental microbes down-regulates ST2 expression in children and thereby protects them against allergies

Written as manuscript for publication

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Abstract

Exposure to microbial components protects children against the development of allergic disorders. But the protective impact of microbes on children's immune system is only poorly understood. Here we investigated the role of ST2, a molecule crucial in regulating innate and T helper cell mediated immune responses, as link between exposure to environmental microbes and development of allergies in children of the European cross-sectional PARSIFAL study. We found that ST2 expression was decreased if children were exposed to microbial components and this, in turn, was associated with reduced T helper cell activation, lower IgE levels in serums, and fewer incidences of allergic diseases. Therefore, ST2 is crucial participant of the immunological network protecting children against the development of allergies in the context of exposure to environmental microbial components.

Introduction

Living on a farm protects children against the development of allergic diseases[1-3]. Epidemiological studies observed that especially microbial components such as lipopolysaccharides (LPS/endotoxin), an integral part of the outer cell membrane of Gram-negative bacteria, are abundant in farming environments and are associated with the protection of children against allergies[4]. The innate immune system constitutes the first contact to invading microbes and directs the activation of the adaptive immune system via induction of co-stimulatory molecules and release of inflammatory cytokines[5, 6]. Exposure to microbial components in environment shapes the innate immune recognition. Farmers' children express more Toll-like receptors (TLR)[7, 8]. The TLR family constitutes the principal receptors for pathogen recognition of the innate immune system[9, 10]. Furthermore, farming lifestyle reduces the ability of children's blood cells to secrete inflammatory cytokines[4].

To avoid effusive inflammatory responses, diverse molecules tightly control the TLR activation pathway[11]. The expression of some of these regulatory molecules is induced by exposure to microbial components leading to a reduced inflammatory response to subsequent stimulation[12-14]. This process is known as endotoxin tolerance[15]. ST2 has been described as such regulator. ST2 (also known as T1, Fit-1, or DER4) is a type I transmembrane protein with three extracellular immunoglobulin-like domains and an intracellular TIR (Toll/interleukin-1 receptor) domain[16, 17]. A soluble form of ST2 exists, too. It is generated by alternative splicing and is present in normal human serum. ST2-deficient mouse macrophages produce elevated amounts of cytokines after stimulation of TLR2, TLR4, and TLR9 but not of TLR3. ST2 achieves its inhibitory effects through sequestration of MyD88 (myeloid differentiation primary-response protein 88) and TIRAP (TIR-associated protein), two adaptor

molecules essential for the activation of the singling cascade of TLR[18-20]. ST2 is induced in macrophages or monocytes after stimulating with TLR4 ligand LPS or with inflammatory cytokines for 4 hours *in vitro,* turning ST2 into a negative feedback inhibitor necessary for endotoxin tolerance[20, 21].

Soluble ST2 is enhanced in serum of patients with inflammatory diseases like asthma but also of autoimmunity indicating an influence of ST2 on T helper cell differentiation[22-24]. T helper cells differentiate into T helper cell type 1 (Th-1), Th-2, or T regulatory cells (Treg) depending on the cytokines present. Th-1 response is associated with autoimmunity, Th-2 with allergic diseases, Treg with a silencing of Th-1 and Th-2 responses[25, 26]. ST2 has an important role in Th-2 effector function and interleukin (IL)-33, a ligand of ST2, induces Th-2 associated cytokine secretion[27-30]. Furthermore, expression of the ST2 gene is regulated by the Th-2 transcription factor GATA-3[31].

The functions attributed to ST2, negative feedback inhibitor of the innate immune system and its role in boosting Th-2 responses, are an immunological contradiction. LPS tolerance is associated with reduced risk to develop allergies, while Th-2 cells promote these diseases. Therefore, we investigated the role of ST2 in children. We assessed the expression of the human ST2 gene in blood leukocytes of children living in areas with various endotoxin levels. Furthermore, we correlated the expression of ST2 gene with the gene expression of markers of T helper cells, with the amount of IgE in serum and with allergic symptoms of children. We found that the expression of the ST2 gene was reduced in children exposed to high levels of endotoxin. Furthermore, the ST2 gene expression was associated with the expression Th-1, Th-2, and Treg cells marker genes. The expression of the ST2 gene was also induced in children with elevated levels of IgE in serum and in children suffering from allergic diseases.

Methods

Population and questionnaires. We assessed gene expression of ST2 and T helper cell markers in the Swiss branch of the PARSIFAL study[32]. RNA samples were collected from 195 farm and 127 reference children (95.3% of children who provided blood samples) to analyze gene expression in their blood leukocytes[8]. The questions on health outcomes were derived from the internationally validated International Study of Asthma and Allergies in Childhood II[33] questionnaire and the Allergy and Endotoxin study[34], respectively. We considered children who had a doctor's diagnosis of asthma and hay fever[8].

Measurement of total and allergen-specific serum IgE levels. Total and allergen-specific IgE for common inhalant (Phadiatop) and food allergens (fx5; Pharmacia CAP

System; Pharmacia Diagnostics AB) was measured in serum. We used for atopic sensitization a cut-off value of 0.35 kU/L or greater[8].

Measurement of IgG, IgA, and IgM serum levels. The serum levels of IgG, IgA, and IgM of children were determined by nephelometric measurements using the Beckman Array® 360 System with the Beckman Reagent Test Packs (Beckman Coulter).

Measurement of endotoxin. Endotoxin was measured in dust samples of mattresses of 83.9% of children with complete gene expression data (n = 270) with the kinetic chromogenic Limulus Amebocyte Lysate test (Bio Whittaker) and expressed as amount of endotoxin per gram dust[8, 35].

RT-PCR and quantitative real-time PCR (TaqMan®). The total RNA was isolated as described[8] and stored at minus 80°C. For reverse transcription (RT) of RNA we used 300ng of total RNA in a final volume of 30µl and added adequate amounts of TaqMan® Reverse Transcription Reagents (Applied Biosystems). Quantitative real-time PCR was performed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems) using the TaqMan® low density array (LDA) system of Applied Biosystems. The determined gene expression values were normalized to the parallel measured endogenous control 18S rRNA. We analyzed the data with the comparative Ct method according to the manufacturer's instructions (Applied Biosystem).

Results

To investigate whether ST2 is involved in mediating endotoxin tolerance *in vivo*, we assessed the expression of the ST2 gene in blood leukocytes of children of a Swiss subsample of the PARSIFAL (Prevention of Allergy Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle) study by quantitative real-time PCR[32]. Furthermore, we measured the endotoxin concentration in dust samples collected of children's mattresses. We found negative correlations between endotoxin in mattress and the expression of the ST2 gene (Geometric mean ration: 0.56 ($p<0.01$) of the 2. tertile related to the 1. tertile; 0.78 ($p>0.1$) of the 3. tertile related to the 1. tertile).

Next, we investigated an association of the ST2 expression with T helper cell differentiation. We related the expression of the ST2 gene to the gene expression of T cell transcription factors T-bet, GATA-3, and FOXP3 measured by quantitative real-time PCR. Each transcription factor is specific for a T cell phenotype: T-bet for Th-1, GATA-3 for Th-2, and FOXP3 for Treg cells. We observed positive associations between the expression of the ST2 gene and the expression of all T cell transcription factor genes (Figure 1).

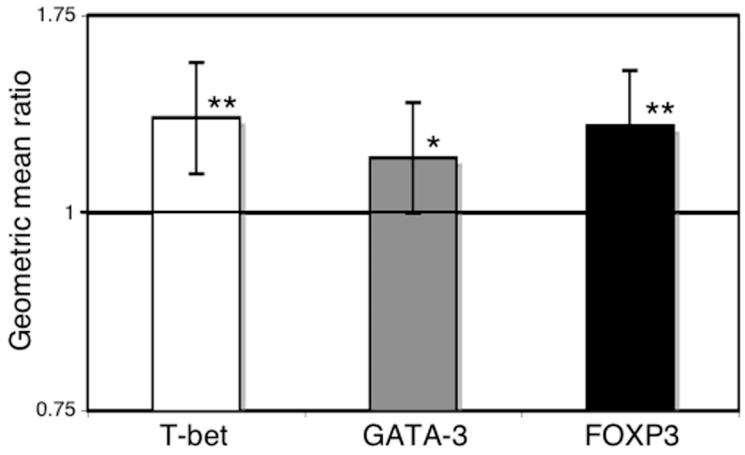

FIGURE 1. Expression of T helper cell marker genes related to the expression the ST2 gene in children's blood leukocytes. We related the expression of the T-bet (white bar), the GATA-3 (grey bar), and the FOXP3 (black bar) gene to the expression of the ST2 gene. Measure of effect size: geometric means rations and 95% confidence intervals; all estimates adjusted for farming status, sex, age, and parental history of atopy; *: $0.05 > p > 0.01$, **: $p < 0.01$.

Since soluble ST2 serum levels were enhanced in patients with asthma, we assessed a correlation between the expression of the ST2 gene in children's blood leukocytes and total immunoglobulin levels in serums. Furthermore, we related the expression of the ST2 gene to the level of specific IgE directed against common inhalant and food allergens and to the incidence of allergic health symptoms of children (Table 1). We found that the ST2 gene expression correlated with enhanced total and allergen-specific IgE serum levels, while there was no association between the expression of the ST2 gene and levels of IgM, IgA, and IgG. Furthermore, the ST2 gene expression correlated with the incidence of asthma and hay fever symptoms in children.

TABLE 1. **Association between ST2 gene expression and Ig serum levels, asthma and hay fever symptoms**

Ig/allergic disease	Odds ratios
total IgM	1.07 (0.48-2.39)
total IgA	1.03 (0.52-2.05)
total IgG	0.99 (0.93-1-06)
total IgE	1.25 (1.13-1.38)**
specific IgE	1.49 (1.2-1.84)**
asthma	1.48 (1.05-2.1)*
hay fever	1.59 (1.06-2.37)*

Values for total IgM, IgA, IgG, IgE geometric means ratios, for specific IgE, asthma, hay fever odds ratios and 95% confidence intervals; adjusted for farming status, sex, age, and parental history of atopy; *: $0.05 > p > 0.01$, **: $p < 0.01$

Discussion

Living of children in areas with elevated levels of microbial components in the environment was associated with reduced expression of ST2. This, in turn, correlated with reduced levels of T helper cells, lower levels of IgE, and less incidence of allergic disorders. Therefore, ST2 represents another immunological mechanism, how exposure to microbes in the environment protects children against the development of allergies.

A role of ST2 in mediating endotoxin tolerance in children via negative regulation of the TLR signaling cascade is implausible, because such a regulator has to be induced through exposure to environmental microbial components as we found it for the suppressor of cytokine signaling (SOCS)-1 protein. It seems that ST2, up-regulated shortly after administration of bacterial components, may regulate TLR signaling cascade in an early phase[20, 21]. Chronic exposure to microbes, as it was the case for the children of our study, reduced the ST2 expression. The observation that ST2-deficient mice were no more susceptible to LPS shock than wild-type mice, make a role of ST2 in mediating endotoxin tolerance more implausible[11, 20]. Nevertheless, our experimental setup failed to fully proof this hypothesis, since we did not perform re-stimulation experiments to assess the responsiveness of children's white blood cells.

ST2 was involved in non-phenotype specific T helper cell activation as shown by positive correlations between ST2 expression and the expression of T helper cell transcription factors. These results stand in contrast to observations that ST2 selectively promotes Th-2 differentiation. But our findings were supported by studies describing enhanced ST2 levels in patients suffering from Th-1 associated autoimmune diseases as well as from Th-2 associated allergic diseases[22-24].

The hygiene hypothesis was emerged after epidemiological studies observed an association between improved hygienic conditions of western countries and the increased incidence of allergic and autoimmune diseases[36]. Besides of the SOCS-1 protein, ST2 is already the second molecule, we described to be a participant in the network constituting the immunological basis of hygiene hypothesis although the functions of these molecules are different. The SOCS-1 protein mediates protection against allergies and autoimmunity of children via regulating the innate immune response, while ST2 seems to be involved in regulation of T helper cell activity.

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CHAPTER 5

MHC class II molecules enhance Toll-like receptor 2-mediated innate immune responses

Written as manuscript for publication

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Introductory paragraph

Major histocompatibility complex (MHC) class II molecules play crucial roles in immune activation by presenting foreign peptides to antigen-specific T helper cells and thereby inducing adaptive immune responses. Although adaptive immunity is a highly effective defense system, it takes several days to become fully operational and needs to be triggered by danger-signals generated during the preceding innate immune response. Toll-like receptors (TLR) recognize conserved molecular patterns of microbes, initiate rapid anti-microbial responses protecting the host during the fist days of infection[1, 2], and generate danger-signals required for activation of the adaptive immune system[3]. A role of MHC class II molecules in initiating innate immune responses has so far not been documented. Here we show that MHC class II molecules synergize with TLR2 in inducing an innate immune response. We found that co-expression of MHC class II molecules and TLR2 leads to enhanced production of the anti-microbial peptide human β-defensin-2 and increased activation of the transcription factor NF-κB after treatment with bacterial lipoproteins. Furthermore, we show that MHC class II molecules are physically associated with TLR2 in lipid raft domains of the cell membrane. These results demonstrate that MHC class II molecules are, in addition to their central role in adaptive immunity, also implicated in generating optimal innate immune responses.

Results and Discussion

To assess a potential role of MHC class II molecules in the innate immune response we compared expression of the antimicrobial peptide human β-defensin (hBD)-2 in transfected cells differing only with respect to their expression of HLA-DR, which is the most abundant MHC class II molecule. The human embryonic kidney (HEK) 293 cell line expresses neither MHC class II molecules nor TLR2, as judged by flow cytometry. These cells were transfected stably with TLR2 and/or HLA-DR1, stimulated with the synthetic TLR2-ligand bacterial lipoprotein (Pam3CysSKKKK, sBLP), and assessed by quantitative real-time PCR for their expression of the hBD-2 gene (Figure 1a). In agreement with previous reports[4, 5], expression of TLR2 alone led to an increase in hBD-2 production, however, only a slight one. Expression of HLA-DR1 together with TLR2 markedly enhanced production of the antimicrobial peptide. Expression of HLA-DR1 alone was not sufficient to confer responsiveness to sBLP.

TLR-triggered cellular responses generally involve NF-κB activation[2]. We therefore, examined whether NF-κB was involved in the enhancement of the innate immune response observed in cells co-expressing TLR2 and HLA-DR1. Using a NF-κB-sensitive reporter plasmid as the readout system we found that NF-κB activation paralleled the induction of hBD-2 expression, except that HLA-DR1 alone supported a low level of sBLP-independent

NF-κB activity (Figure 1b). Moreover, the induction of hBD-2 by sBLP through TLR2/HLA-DR1 was down regulated in a dose-dependent manner by the NF-κB inhibitor peptide NBD (Figure 1c). The expression of HLA-DR1 together with TLR2, therefore, augments TLR2-ligand triggered, NF-κB-mediated cellular activation.

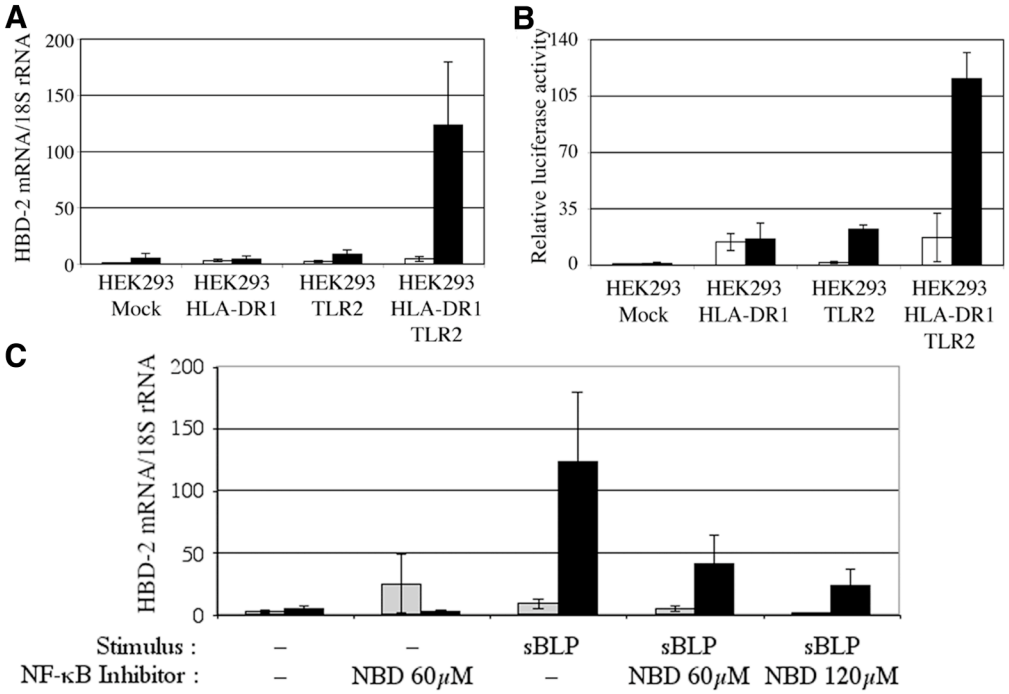

FIGURE 1. Expression of HLA-DR1 enhances TLR2 dependent activation of HEK293 cells. (A,B) Co-expression of HLA-DR1 in $TLR2^+$ HEK293 cells leads to enhanced hBD-2 expression (n=5) and NF-κB activity (n=3) after stimulation with sBLP (black bars) compared to untreated cells (white bars). (C) The NBD peptide inhibits NF-κB mediated hBD-2 expression after stimulation with sBLP in $TLR2^+$ (grey bars) and $TLR2^+$/$HLA\text{-}DR1^+$ (black bars) HEK293 cells (n=3). Error bars represent standard errors.

Next, we evaluated the contribution of MHC class II molecules to innate immune response in another experimental model. Since the expression of inducible NO synthase (iNOS) has been reported to be induced by TLR2 engagement in mice[6], we compared expression of the iNOS gene in lung tissues isolated from wild type (wt) and MHC class II knock-out (ko) mice of the same genetic background (C57BL/6) following treatment with sBLP[7]. The response of the MHC class II ko tissue was markedly reduced compared to that of the wild-type tissue (Figure 2). This *ex vivo* system, thus, reproduces the results obtained with the HEK293 cells.

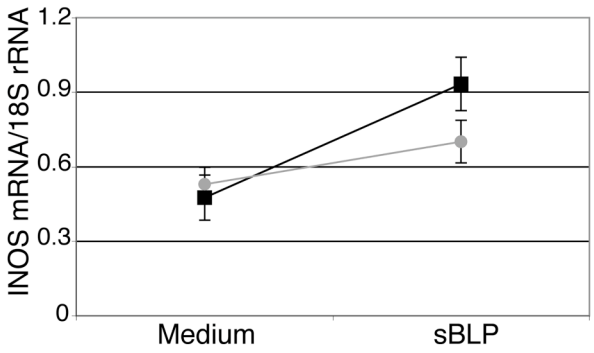

FIGURE 2. Lung tissues from MHC class II ko mice exhibit impaired responsiveness to sBLP. Inducible NO synthase mRNA expression in lung tissue from MHC class II knock-out mice (grey curve; n=5) and wt control mice (black curve; n=5) after *ex vivo* stimulation with sBLP. Error bars represent standard errors.

We then assessed whether the functional synergism between TLR2 and MHC class II molecules might involve their physical interaction. We found that recombinant radioactively labeled TLR2 can be co-precipitated with HLA-DR1 (Figure 3). MHC class II molecules are localized in lipid raft domains of the cell membrane and these membrane domains are essential for efficient signaling processes[8-11]. A role for lipid rafts in TLR-signaling has previously been reported for TLR4[12]. We found that methyl-β-cyclodextrin (MCD) mediated destruction of lipid raft domains inhibited sBLP-triggered expression of the hBD-2 gene in a dose dependent manner in HLA-DR1^{+}/TLR2^{+} cells (Figure 4). Moreover, immunofluorescence microscopy revealed that HLA-DR1 and TLR2 co-localize in lipid raft domains (Figure 5).

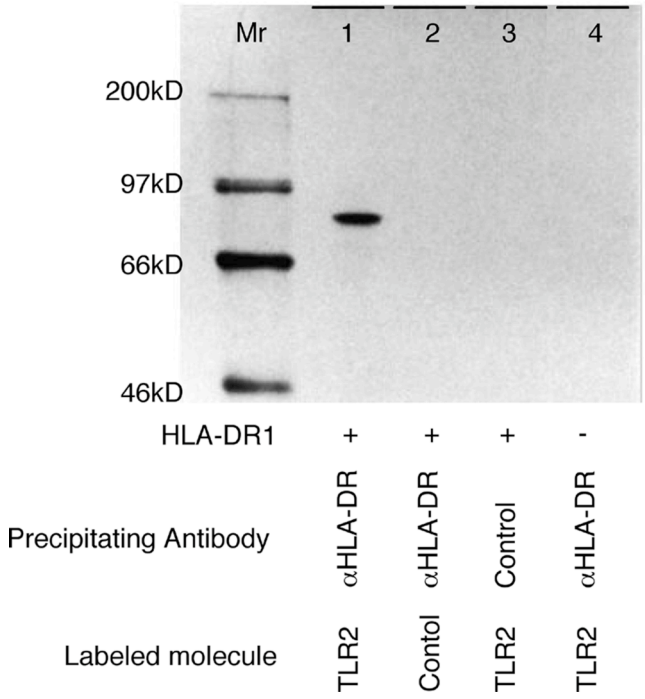

FIGURE 3. TLR2 and HLA-DR1 interact physically. Co-precipitation of labeled recombinant TLR2 (lane 1, 3, 4) or a control protein (lane 2, luciferase) with HLA-DR1 molecules purified by immunoprecipitation with anti HLA-DR antibodies (lane 1, 2, 4) or control antibodies (lane 3) from cell lysates of HLA-DR1 positive (lane 1, 2, 3) or negative (lane 4) HEK293 cells. A ^{14}C-labeled molecular weight marker (in kD) is shown in the left lane.

Our results reveal a highly novel and unexpected function of MHC class II molecules. We show here that MHC class II molecules can play a key role in innate immunity by enhancing TLR-mediated cellular activation. The induction of MHC class II expression by TLR agonists[13, 14], thus, not only enables effective antigen presentation for the activation of adaptive immunity, but also functions as a positive feedback mechanism that enhances TLR-mediated responses. The innate immune response is a tightly regulated process involving numerous stimulatory and inhibitory molecules[15]. Over-reaction of the innate immune system may lead to chronic inflammation, allergy, and autoimmunity. Insufficient activation results in both inadequate protection during the first days of infection and in inefficient or inappropriate activation of the adaptive immune system, potentially leading to disease or death. We have identified MHC class II molecules as novel participants in the complex regulatory mechanisms that control the efficiency of innate immune responses.

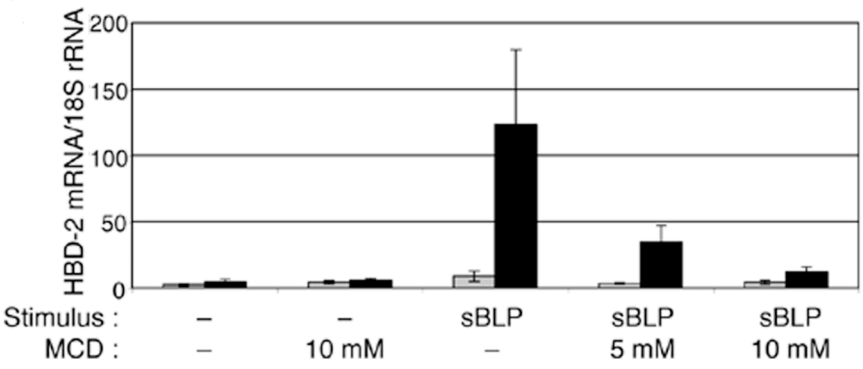

FIGURE 4. Enhanced responsiveness of HLA-DR1$^+$/TLR2$^+$ HEK293 cells is dependent on lipid raft domains. Treatment of TLR2$^+$ (grey bars; n=3) or HLA-DR1$^+$/TLR2$^+$ (black bars; n=3) HEK293 cells with MCD, a lipid raft-destroying agent, prior to stimulation with sBLP leads to impaired hBD-2 gene expression. Error bars represent standard errors.

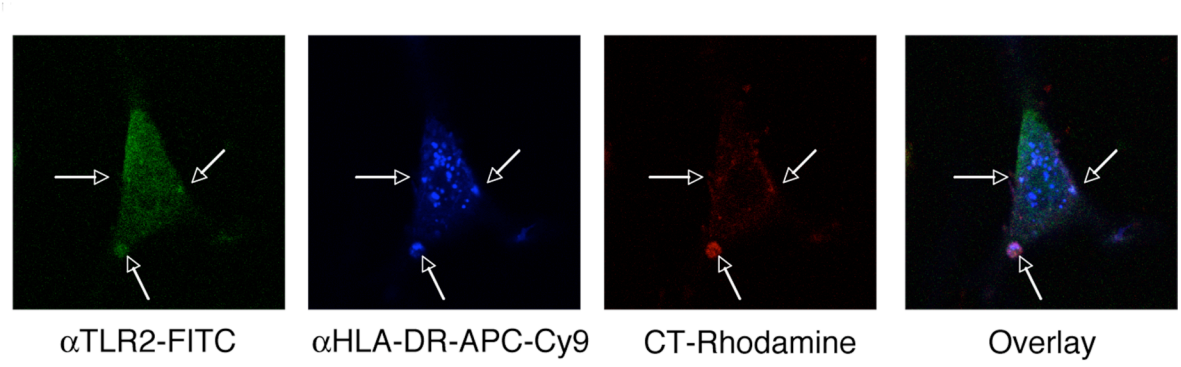

FIGURE 5. TLR2 and MHC class II molecules co-localize in lipid raft domains. Immunofluorescence microscopy was used to show that TLR2 (αTLR2-FITC; green) and HLA-DR1 (αHLA-DR-APC-Cy9; blue) co-localize in lipid raft domains (CT-rhodamine; red) of HLA-DR1$^+$/TLR2$^+$ HEK293 cells (indicated by the white arrows). Pictures are representatives of at least five experiments.

Methods

Cell lines and media. The human embryonic kidney cell line (HEK293) was obtained from the American Type Collection Culture (ATCC). HEK293 cells transfected with HLA-DR1 (α-, β-, and invariant chain) were a gift from Dr. J. Neffjes (Netherlands Cancer Institute). These cells were stably transfected with an empty pCEP4 plasmid (Invitrogen) or a pUNO-hTLR2 plasmid (InvivoGen) using the TransIT-293 Transfection Reagents (Mirus). Cells were grown in DMEM (Gibco BRL) supplemented with 10% low endotoxin FBS (Hyclone) and 1% of an antibiotic-antimycotic solution (Gibco BRL). Depending on the resistance genes present, medium was supplemented with the additional selection antibiotics G418, Hygromycin (Roche Diagnostics), Ovabain (Sigma), or Blasticidin (InvivoGen).

NF-κB reporter gene assay. Cells were seeded in 6-well plates ($2x10^5$ cells/well) and incubated at 37°C in 5% CO_2. When at least 80% confluent, cells were transfected with pNF-κB-Luc (NF-κB dependent luciferase reporter plasmid, 1.25µg/ml, Clonetech) and pCMV-lacZ (CMV-promoter controlled β-galactosidase plasmid, 0.6µg/ml, a gift from Dr. J.-P. Hossle, Zürich, Switzerland) using TransFectin Lipid Reagent (BioRad). 48h after transfection the cells were stimulated for 6h with synthetic bacterial lipoprotein (1µg/ml, sBLP, Pam3Cys-SKKKK, EMC microcollections). After cell lysis, luciferase activity was measured in counts per minute (CPM) using a luciferase assay system (Promega) and a TD-20/20 Luminometer (Turner designs). Values were corrected for transfection efficiencies (divided by the lacZ-activity determined as the absorption at 420nm).

Cell stimulation and total RNA extraction. Cells were grown in 6-well plates at 37°C in 5% CO_2 until they were 80% confluent, and then stimulated for 6h with sBLP (1µg/ml) or sBLP in combination with various concentrations of the NBD peptide (Alexis Biochemicals) or Methyl-β-cyclodextrin (MCD, Sigma-Aldrich). NBD peptide or MCD were added 30 or 10 minutes before sBLP, respectively. The QIAmp RNA Blood Mini Kit (Qiagen) supplemented with RNase-free DNase (Qiagen) was used for total RNA isolation.

Ex vivo mouse experiments. Experiments were performed with wild type (Harlan) and MHC class II deficient[7] C57BL/6 mice. Lung tissues were stimulated for 4h with 10µg/ml sBLP in RPMI 1640 (Gibco BRL) containing 10% FBS. Tissues were homogenized using a rotor-stator tissue homogenizer and total RNA was isolated with the QIAmp RNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions.

Quantitative real-time PCR. Reverse transcription and quantitative real-time PCR were performed with reagents from Applied Biosystems according to the manufacturer's instructions. Quantitative real-time PCR was performed with an ABI Prism 7700 Sequence Detection SystemTM (Applied Biosystems). For hBD-2 mRNA, primers were 5'-GAGGAGGCCAAGAAGCTGC-3' (300nM) and 5'-CGCACGTCTCTGATGAGGG-3' (300nM), and the probe was 5'-TGGCTGATGCGGATTCAGAAAGGG-3' (250nM). For 18S rRNA primers were 5'-AGTCCCTGCCCTTTGTACACA-3' (200nM) and 5'-GATCCGAGGGCCTCACTAAAC-3' (200nM), and the probe was 5'-CGCCCGTCGCTACTACCGATTGG-3' (250nM). All oligonucleotides were synthesized by Microsynth. A pre-developed assay (Applied Biosystems; Mm00440485_m1) was used to measure expression of the inducible nitric oxide synthase gene. Results were normalized relative to 18S rRNA using the Comparative (ΔΔCt) method according to the manufacturer's instructions (Applied Biosystems).

Co-immunoprecipitations. Immunoprecipitations were performed using the 'Cellular Labelling and Immuoprecipitation Kit' according to the manufacturer's instructions (Roche

Diagnostics). HEK293 cells were collected in lysis buffer, sonificated, and insoluble material was removed by centrifugation. To eliminate non-specific binding, supernatants were incubated with 50µl protein A agarose and cleared by centrifugation. Immunoprecipitation was then performed by mixing 1µg of unlabeled anti-HLA-DR1 antibodies with 40µl of the pre-cleared supernatant and 50µl protein A-agarose. The immunoprecipitates were collected by centrifugation, washed and dissolved in 50mM TBS (pH 7.5). Recombinant radio-labelled proteins were synthesized using the TNT T7 Quick Coupled Transcription/Translation System (Promega) and pBluescript KS (-) plasmids expressing TLR2 or luciferase. The recombinant molecules were added to the immunoprecipitated samples and complexes were collected by centrifugation, washed, and analyzed by SDS-PAGE.

Immunofluorescence microscopy. HEK293 cells expressing HLA-DR1 and TLR2 were grown overnight on coverslips and fixed for 15 min. with 3% paraformaldehyde. Cells were stained by sequential 45 min. incubations with rhodamin-labelled cholera toxin B subunit (0.5µg/ml; List Biological Laboratories) to visualize the lipid raft domains, a FITC anti-human TLR2 monoclonal antibody (T2.1; eBioscience), and an APC-Cy9 anti-human HLA-DR monoclonal antibody (L243; BD Biosciences). Cells were washed three times with PBS between each staining step. Cells were mounted in Glycergel (DakoCytomation) and observed with a Leica TCS SL confocal microscope using the 63x/1.2 W CORR PL APO objective (Leica).

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CHAPTER 6

Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children

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Abstract

BACKGROUND: There is increasing evidence that environmental exposures determining childhood illnesses operate early in life. Prenatal exposure to a farming environment through the mother might also play an important role. OBJECTIVE: We sought to investigate the role of maternal exposures to environments rich in microbial compounds for the development of atopic sensitization, asthma, and corresponding alterations in the innate immune system in offspring. METHODS: In the children of the cross-sectional Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Life Style study, asthma and atopy were assessed by means of standardized questionnaires (n = 8263) and serum IgE measurements (n = 2086). In a subsample (n = 322) gene expression of Toll-like receptors (TLR2 and TLR4) and CD14 was assessed. Maternal exposures were defined through questionnaire information. RESULTS: Both atopic sensitization (adjusted odds ratio, 0.58; 95% CI, 0.39-0.86) and the gene expression of receptors of innate immunity were strongly determined by maternal exposure to stables during pregnancy, whereas current exposures had much weaker or no effects. A dose-response relation was found between the extent of upregulation of these genes and the number of different farm animal species the mother had encountered in her pregnancy. Each additional farm animal species increased the expression of TLR2, TLR4, and CD14 by a factor of 1.16 (95% CI, 1.07-1.26), 1.12 (95% CI, 1.04-1.2), and 1.10 (95% CI, 1.03-1.23), respectively. CONCLUSION: Maternal exposure to an environment rich in microbial compounds might protect against the development of atopic sensitization and lead to upregulation of receptors of the innate immune system. The underlying mechanisms potentially operating through the intrauterine milieu or epigenetic inheritance await further elucidation. CLINICAL IMPLICATIONS: When assessing risk factors of allergies in an infant's medical history, attention must also be paid to environmental exposures affecting the mother.

Introduction

Childhood asthma and allergies manifest in the first years of life[1, 2]. Environmental exposures implicated in the inception of these illnesses must therefore occur even earlier (ie, before the manifestation of the first symptoms). In recent years, a number of studies have shown that exposures in the first 12 to 24 months of life might indeed determine the development of childhood asthma and allergies[1, 3-5]. The temporal sequence of events might, however, be traced back to prenatal exposures, namely exposures of the mother. Active smoking during pregnancy[6]; exposure of the pregnant mother to antibiotics[7], paracetamol[8], and allergens[9, 10]; and the effect of such exposure on the fetus have been investigated in some

detail. Still, little is known about the effects of maternal exposure to microbial compounds in the environment on the development of atopy and asthma in offspring.

A deviated humoral immune response resulting in the production of IgE rather than IgG antibodies is the common feature of atopic diseases. The regulation of the humoral response by B cells depends on T effector cells. In turn, T-cell function is regulated by mediators of the innate immune system. Although it has been shown that adaptive immune responses can be shaped in utero[11], little is known about potential prenatal determinants of the innate immune response and its relation to the development of atopy and asthma.

The aim of the present study was to investigate the role of exposures of the mother and the child, respectively, to environments rich in microbial burden for the development of atopy and asthma, as well as the gene expression of receptors of the innate immune response in the offspring.

Methods

Population and study areas. The cross-sectional Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Life Style (PARSIFAL) study aimed at studying the determinants of childhood asthma and allergies in farming and anthroposophic populations, as described previously[12]. A child who lived on a farm and whose family ran the farm was considered a farm child. Other children were termed farm reference children. The present analyses focus on 2823 farm and 5440 farm reference children aged 5 to 13 years from rural areas of Austria, Germany, the Netherlands, Sweden, and Switzerland. In Sweden farmers with children were identified from the Farming Registry at the National Bureau of Statistics, and farm reference children were randomly selected from the population registry among children living in the same area. In Austria all farm children of a given school class were chosen by teachers who had a good knowledge of the region, and reference children were randomly selected from the same schools by the local study group. In the other countries children were randomly selected from schools in areas known to have a high percentage of farmers.

The study was approved by the ethical boards of the 5 study centers, and written informed consent was obtained from the children's parents or guardians for questionnaires, blood sampling, and genetic analyses.

Questionnaires. The questionnaire comprised questions on sociodemographic background, parents' atopic diseases, family, and housing characteristics. In addition, information on the child's farm activities, as well as the mother's farm exposures during pregnancy, were available. Questions related to the child's contact with different farm animals

(during the first year of life or later), consumption of farm milk (during the first year of life or current), and regular stable or barn visits and helping with haying at present. Activities occurring at least weekly were defined as occurring regularly. Maternal exposure included contact to different farm animal species during pregnancy and regularly working in stables (sheep shelters, hog, cow, and chicken houses) during pregnancy and lactation. The questionnaire asked for contact with 6 different farm animal species (cows, pigs, sheep, horses, goats, and poultry), and these were summed.

Questions on health outcomes and farm exposures were derived from the internationally validated International Study of Asthma and Allergies in Childhood II[13] questionnaire and the Allergy and Endotoxin study[14], respectively. Children were considered to have current rhinoconjunctivitis symptoms if sneezing, runny nose, stuffy nose, and itchy eyes were reported in the last 12 months without the child having a cold at the same time. Current wheezing was defined as at least one episode of wheezing during the last 12 months. Children reported to have had symptoms of seasonal rhinoconjunctivitis in the last 12 months were defined as symptomatic, whereas children with a report of a physician's diagnosis of hay fever were considered to have a physician's diagnosis of seasonal rhinoconjunctivitis. Children with a report of a physician's diagnosis of asthma or of obstructive bronchitis more than once in their lifetime were considered to have a physician's diagnosis of asthma.

Measurement of allergen-specific serum IgE levels. In Austria, the Netherlands, and Sweden all children whose parents had consented to blood sampling were invited to a physical examination with blood sampling. In Germany and Switzerland only a random sample of those who consented were invited because of the comparatively large number of children included in these countries[12]. This subsample of children with blood analysis (n = 2086) did not significantly differ from the whole sample in regard to disease frequencies or family history of atopic disease (data not shown).

Allergen-specific IgE for common inhalant (Phadiatop) and food allergens (fx5; Pharmacia CAP System; Pharmacia Diagnostics AB, Uppsala, Sweden) was measured in serum. Two cut-off values for atopic sensitization were used: IgE values of 0.35 kU/L or greater and 3.5 kU/L or greater for either inhalant or food allergens.

Expression of Toll-like receptors. For the Swiss branch of the PARSIFAL study, RNA samples were collected from 195 farm and 127 reference children (95.3% of children who provided blood samples) to analyze gene expression of innate immunity receptors. Children with available RNA samples did not differ significantly from the total Swiss PARSIFAL population with respect to farm exposures and health outcomes (data not shown).

The total RNA was isolated with the QIAmp RNA Blood Mini Kit (Qiagen, Hilden, Germany) supplemented with RNase-free DNase (Qiagen). Quantitative real-time PCR (TaqMan; Applied Biosystems, Foster City, Calif) was performed, as described elsewhere[15]. In brief, primers and probes were designed by using the primer design software Primer Express (Applied Biosystems). Optimal concentrations for primers and probes were determined according to the manufacturer's instructions. The reactions for the target and the endogenous control were performed in separate tubes. All PCR reactions were analyzed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Experiments assessing background signals were performed for every assay by running the reaction without templates ("no template controls"). No amplification was observed in any of these "no template controls," indicating that there was neither contamination nor unspecific fluorescence (see Fig E1 in the Online Repository at www.jacionline.org). The data are presented as normalized values (the amount of mRNA of the target molecule divided by the amount of mRNA of the endogenous control [18s rRNA]).

Measurement of endotoxin and extracellular polysaccharide in dust samples. Endotoxin and fungal extracellular polysaccharide (EPS) were measured in mattress dust samples of 83.9% of children with complete gene expression data (n = 270). Sampling and detection methods are described elsewhere[16]. In brief, endotoxin was measured with the kinetic chromogenic Limulus Amebocyte Lysate test (Bio Whittaker, Walkersville, Md) and EPS with a specific sandwich enzyme immunoassay for EPS of Aspergillus and Penicillium species[17].

Statistical analysis. Statistical analysis was performed with SAS 9.1.3 (The SAS Institute, Cary, NC) and Stata/SE 8.2 (StataCorp LP, College Station, Tex) software. Odds ratios (ORs) or geometric means ratios and 95% CIs were calculated in multiple logistic regression analysis.

In preliminary logistic analyses the associations of atopic sensitization, atopic symptoms, or diseases and exposure to farm characteristics were explored and adjusted for predefined covariates (age, sex, parental education, maternal and paternal atopy, number of older siblings, exposure to pets, and study center). We included farm characteristics that best predicted being a farm child, namely the child's current exposure to a farming lifestyle (regular stable or barn visits or helping with haying), the child's exposure to farm animals (ever), predominant consumption of farm milk (ever), and regular maternal work in stables during pregnancy. In the final regression model all variables were mutually adjusted. The independent effects of maternal work in stables during pregnancy and lactation were investigated to evaluate the effect of timing of the exposure.

Because the distribution of gene expression levels of the CD14, Toll-like receptor (TLR) 2, and TLR4 genes was skewed, these variables were log-transformed, resulting in a good approximation to the normal distribution (data not shown). For farm and farm reference children, these log-transformed values were compared by using the t test, and crude geometric means with their 95% CIs were computed. Multiple regression models adjusting for the same covariates as mentioned above were used to explore the associations between gene expressions and farm-related exposure measures. Results were expressed as adjusted geometric means ratios. In addition, adjusted geometric means of normalized gene expression were calculated for different levels of specific exposure variables (eg, the number of farm animal species the mother was exposed to during pregnancy).

In further analyses the effect of additional exposures during intervening years (5-13 years) that might influence atopic sensitization or gene expression were tested. In turn, the following variables were included in the regression models: worm infestations ever, respiratory infections during the first 2 years of life, day-care attendance, prescription of antibiotics or antipyretics before or after the first year of life, and history of measles, rubella, mumps, pertussis, or mononucleosis.

Results

Of the 11,969 invited farm and farm reference children, 8402 (70%) returned the questionnaires. A total of 139 children were excluded because of missing values for sex and age or because they did not meet the age criteria of 5 to 13 years.

The prevalence of all outcomes (atopic sensitization, rhinoconjunctivitis symptoms and physician's diagnosis of seasonal rhinoconjunctivitis, and current wheezing and physician's diagnosis of asthma) was significantly lower in farm children compared with in nonfarm children (see Fig E2 in the Online Repository at www.jacionline.org)[12], despite some heterogeneity across the countries. The most pronounced differences between farm and nonfarm children were seen in Germany.

Factors mediating the farm effect

Determinants of the protective farm effect were assessed in multiple regression models (Table 1). Predominant farm milk consumption was inversely related to the symptoms (P = .079) and diagnosis of seasonal rhinoconjunctivitis (P = .022), asthma (P = .038), and wheezing (P = .065). Additionally, regular contact with farm animals contributed to the protective effect on the physician's diagnosis of seasonal rhinoconjunctivitis (P = .049). Rhinoconjunctivitis symptoms in the last 12 months were also inversely related to frequent

stable or barn visits or help with haying (P = .007). The strongest protective effect, however, was found for maternal stable work during pregnancy on atopic sensitization (OR, 0.58; 95% CI, 0.39-0.86; P = .007). Moreover, this association remained significant after additional adjustment for being a farm child or correction for multiple testing ($P_{Bonferroni}$ = .035).

TABLE 1. **Mutually adjusted ORs for associations of farm-related exposures with health outcomes**

	Atopic sensitization (≥ 3.5 kU/L) (n=285/2086)	Rhinoconjunctivitis symptoms (n=507/8174)	Physician's diagnosis of rhinoconjunctivitis (n=343/8130)	Wheezing (n=552/8169)	Diagnosis of asthma (n=656/8080)
Current farm exposure*	0.96 (0.63-1.46) p=0.854	0.63 (0.45-0.88) p=0.007	0.66 (0.41-1.07) p=0.090	0.88 (0.65-1.19) p=0.403	0.82 (0.62-1.09) p=0.172
Regular contact to farm animals ever	0.76 (0.51-1.15) p=0.194	0.87 (0.67-1.14) p=0.321	0.69 (0.47-1.00) p=0.049	0.97 (0.75-1.26) p=0.822	0.94 (0.75-1.19) p=0.629
Farm milk consumption ever	0.76 (0.52-1.11) p=0162	0.77 (0.58-1.03) p=0.079	0.63 (0.42-0.93) p=0.022	0.77 (0.58-1.02) p=0.065	0.76 (0.59-0.99) p=0.038
Stable exposure in pregnancy†	0.58 (0.39-0.86) p=0.007	0.74 (0.50-1.09) p=0.126	0.77 (0.44-1.36) p=0.371	0.76 (0.54-1.07) p=0.120	0.86 (0.63-1.16) p=0.325

ORs are given with 95% CIs in parentheses and P values. The models are adjusted for age, sex, family history of atopy, parental education, environmental tobacco smoking, maternal smoking during pregnancy, number of older siblings, contact with pets ever,

* Current regular exposure to stable or barn or regular participation in haying.

† Mother worked regularly in stable during pregnancy.

Maternal stable exposure during pregnancy versus lactation

Because 20% of all mothers working in the stable during pregnancy (n = 2184) stopped working in the stable after giving birth to the child, it was possible to disentangle the contributions of pregnancy and lactation, respectively, to the maternal effect. The effect of maternal stable work during lactation tended to be less pronounced (OR, 0.66; 95% CI, 0.45-0.96). It was no longer statistically significant when adjusted for maternal exposure in pregnancy (OR, 0.92; 95% CI, 0.52-1.61). When assessing the effect of timing of first exposure to farm animals on atopic sensitization, adjusted ORs for first contact in pregnancy, first contact in first year of life, first contact between first year and current, and current first contact were 0.36 (95% CI, 0.25-0.51), 0.54 (95% CI, 0.32-0.92), 0.77 (95% CI, 0.49-1.22), and 0.71 (95% CI, 0.43-1.20), respectively.

Variation of the effect on atopic sensitization across countries

We furthermore assessed the effect of maternal stable work during pregnancy on atopic sensitization for each country separately (Figure 1). The protective maternal effect was found in all countries but Sweden (OR, 1.41; 95% CI, 0.50-4.01). We investigated different cut-off definitions in the 4 countries showing a protective maternal effect. Using the detection limit as cut-off (≥0.35 kU/L) yielded a somewhat weaker but still significant effect (OR, 0.73; (95% CI, 0.54-0.995) compared with a cut-off level of 3.5 kU/L or greater (OR, 0.53; 95% CI, 0.35-0.80).

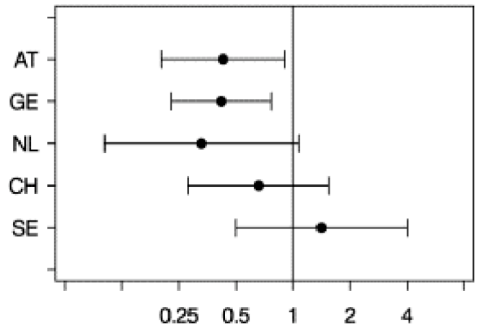

FIGURE 1. Adjusted ORs for maternal work in stables during pregnancy on atopic sensitization across countries. As a cut-off point for atopic sensitization, a value of 3.5 kU/L or greater was used. The ORs are given separately for the following countries: Austria (AT), Germany (GE), the Netherlands (NL), Switzerland (CH), and Sweden (SE).

Association of farm-related exposure with gene expression

Because environmental factors have been shown to correlate with the expression of receptors of the innate immune system, we next investigated the expression of the genes for TLR2, TLR4, and CD14 in the Swiss subpopulation. Geometric means of the expression of these genes were significantly higher among farm children than among farm reference children (TLR2: 0.74 [95% CI, 0.65-0.84] vs 0.49 [95% CI, 0.43-0.56], P < .0001; TLR4: 0.98 [95% CI, 0.88-1.09] vs 0.82 [95% CI, 0.72-0.93], P = .034; CD14: 0.48 [95% CI, 0.41-0.55] vs 0.28 [95% CI, 0.24-0.31], P < .0001), thereby confirming previous data from another farming population[18]. It was, however, not the current exposure to several farm characteristics but rather maternal exposure during pregnancy that was associated with increased gene expression (Table 2). Likewise, current levels of endotoxin and EPS in mattress dust were not correlated with gene expression of their respective receptors (data not shown and Fig E3 in the Online Repository at www.jacionline.org). Day-care attendance, reported childhood infections, worm infestation, and use of antibiotics or antipyretics during intervening years were not associated with gene expression of TLR/CD14 and atopy and did not affect reported point estimates. However, a dose-response relation was found when relating the number of different farm animal species the mother had contact with during pregnancy to gene expression (Figure 2).

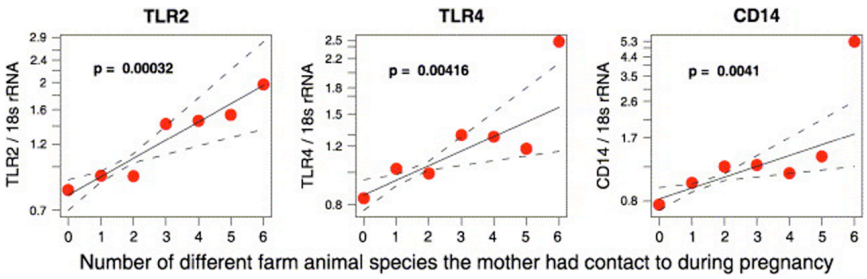

FIGURE 2. The x-axis shows increasing numbers of different farm animal species the mother had contact with during pregnancy (n = 322). The y-axis shows geometric means (dots) of gene expression of TLR2, TLR4, and CD14, respectively, which were normalized by dividing by gene expression of 18s rRNA. In addition, the linear fit of the association (solid line), along with the corresponding 95% CI (dashed line), is displayed. All analyses were adjusted for age, sex, family history of atopy, parental education, environmental tobacco smoking, maternal smoking during pregnancy, number of older siblings, contact with pets ever, child's current exposure to a farming lifestyle, child's exposure to farm animals, and predominant farm milk consumption of the child.

Each additional species of farm animal the mother was exposed to during pregnancy increased the expression of TLR2, TLR4, and CD14 by a factor of 1.16 (95% CI, 1.07-1.26), 1.11 (95% CI, 1.03-1.19), and 1.14 (95% CI, 1.04-1.24), respectively. Neither restricting the analysis to farm children only nor omitting the highest category of contact to farm animal species during pregnancy materially changed the results. In a model including exposure to different animal species during pregnancy and during the first year of life simultaneously, stronger effects were observed for exposure during pregnancy (TLR2, 1.11 [95% CI, 1.01-1.21]; TLR4, 1.05 [95% CI, 0.97-1.14]; CD14, 1.09 [95% CI, 0.99-1.21]) than during the first year of life (TLR2, 1.03 [95% CI, 0.94-1.12]; TLR4: 1.02 [95% CI, 0.95-1.10]; CD14: 1.00 [95% CI, 0.91-1.10]), respectively. The correlation between these 2 variables was moderate (r = 0.69).

TABLE 2. **Mutually adjusted geometric means ratios for associations of farm-related exposures with the expression of the TLR2, TLR4, and CD14 genes (n = 322)**

	TLR2	TLR4	CD14
Current farm exposure*	1.04 (0.69-1.55) p= 0.851	0.93 (0.66-1.3) p= 0.671	1.01 (0.66-1.54) p= 0.964
Regular contact to farm animals ever	1.09 (0.75-1.58) p= 0.650	0.92 (0.67-1.25) p= 0.577	0.97 (0.65-1.43) p= 0.866
Farm milk consumption ever	1.04 (0.77-1.42) p= 0.813	1.06 (0.81-1.4) p= 0.656	1.16 (0.83-1.64) p= 0.385
Stable exposure in pregnancy†	1.44 (1.04-1.98) p= 0.027	1.4 (1.07-1.83) p= 0.015	1.66 (1.18-2.33) p= 0.003

Geometric means ratios are given with 95% CIs in parentheses and P values. The models are adjusted for age, sex, family history of atopy, parental education, environmental tobacco smoking, maternal smoking during pregnancy, number of older siblings, conta

* Current regular exposure to stable or barn or regular participation in haying.

† Mother worked regularly in stable during pregnancy.

Discussion

The present study showed a clear inverse association of a farming environment (farm milk consumption, stable/barn visits, and contact with farm animals) with the prevalence of seasonal rhinoconjunctivitis, symptoms of seasonal rhinoconjunctivitis, and asthma. Maternal exposure to stables in pregnancy was associated with atopic sensitization and the expression of genes for receptors of the innate immune system (ie, TLR2, TLR4, and CD14). A dose-response relation was found between the extent of the upregulation of these genes and the number of different farm animal species the mother had contact with during her pregnancy. However, only longitudinal studies will eventually establish a causal relationship.

The identification of farm milk and stable animal contact as being protective for asthma and seasonal rhinoconjunctivitis is in accordance with the results of the Allergy and Endotoxin study[14]. The much larger size of the PARSIFAL population allowed, however, a more detailed investigation of the relevant time windows of exposure for each outcome. A stronger effect of prenatal (ie, maternal) exposure was seen for atopic sensitization. Moreover, this effect was observed for gene expression of innate immunity receptors. Stable exposure during pregnancy was more relevant than exposure while the mother was breast-feeding, thereby shifting the focus to prenatal exposure. The cross-sectional design of the study might have resulted in recall bias. However, this is unlikely because the outcomes (IgE levels and gene expression) were laboratory parameters not known to the mothers when answering the questionnaires. The maternal effect was independent of the mother's history of atopy and consistent over geographic areas, with the exception of Sweden. In Sweden none of the stables were connected to farmhouses in contrast to the other countries, potentially indicating a different pattern and intensity of exposure. Moreover, the levels of microbial compounds in house dust differ between Sweden and the other countries[16].

Exposure to farm animals and stables has been found to be associated with increased exposure to various microbial products[19, 20]. TLR2 is a receptor for various microbial compounds from gram-positive bacteria and fungi, such as lipopeptides, lipoarabinomannan, lipoteichoic acid, and glucans, whereas TLR4 recognizes LPSs mostly from gram-negative bacteria. CD14 in turn is involved in the recognition of LPSs and other bacterial wall components[21]. It seems therefore likely that the upregulation of these genes with an increasing number of farm animal species the mother was exposed to during pregnancy is attributable to increasing levels, diversified levels, or both of microbial exposure associated with contact with these animals.

By recognizing microbial compounds, TLRs are part of innate immunity, the first line of defense. The innate immune response communicates with the effector cells of the adaptive immune response, which includes the T helper cell populations[22]. Th1 cells favor the

production of IgG antibodies, whereas Th2 cells promote the production of IgE antibodies and thereby the development of atopic diseases. In experimental studies it has been shown that stimulation of the innate immune response might skew the balance toward antiallergic TH1 responses[23, 24]. However, in children of farming environments with high endotoxin exposure, a decreased secretion of both Th1 and Th2 cytokines was observed on restimulation of leukocytes through the TLR4 ligand LPS[25]. This finding suggests that mechanisms other than a Th1/Th2 imbalance might be operational in these environments. T regulatory cells or mechanisms inherent to innate immune signaling, such as LPS tolerance, might play a role[18, 26, 27].

In the present study current exposures to either stables and barns or to measured levels of microbial products indoors were not related to expression of these innate immunity genes. Yet other microbial exposures than the ones assessed might have been of greater importance. However, endotoxin and EPS are specific ligands for TLR4 and TLR2, respectively[21], which makes it improbable that we have missed major environmental signals.

In contrast, maternal contact with increasing numbers of farm animal species, which is likely to parallel microbial exposures, was significantly related to a long-lasting upregulation of the expression of these innate immunity genes, which was still detectable at school age. Similarly, maternal work in stables during pregnancy was associated with decreased production of allergen-specific IgE antibodies, which was again detectable at school age. Thus prenatal environmental influences might affect the long-term development of innate and adaptive immune responses. In turn, the manifestation of organ-specific atopic conditions of the upper and lower respiratory tract might depend on the interplay between the growing and maturing child and his or her environment.

It is unknown how the maternal environment exerts its effect on the offspring. One might speculate that the fetal immune system interacts with a certain cytokine pattern prevailing in the maternal organism[28] or with the antigens themselves[29], implying the establishment of immunologic tolerance. Another attracting explanation might consist in epigenetic inheritance. Differential patterns of gene expression arise during development in utero and are subsequently retained through mitosis. Stable alterations are heritable but do not involve mutations of the DNA itself[30, 31]. Recent data suggest that environmental factors might alter methylation processes, a key element of epigenetic inheritance[30]. Furthermore, there is some evidence suggesting that endotoxin has a role in gene silencing because LPS is a potent inflammogen that stimulates acetylation of histones[32].

The PARSIFAL study does not provide information on the maternal exposure before pregnancy, and therefore we cannot exclude a preconceptional effect. In other words, the mother's earlier exposure to a farming environment might have determined a configuration of

her immune system, which she then passed on to her child. If indeed preconceptional exposures were important, environmental determinants of disease inception would also operate in the parental generation. Recent observations demonstrating a plateau of the prevalences of asthma, seasonal rhinoconjunctivitis, and atopic sensitization after 3 decades[33-35], which approximates a generation, might indirectly support the notion of a generational effect. Furthermore, a transgenerational association of a grandmother's smoking with her grandchildren's risk for asthma has recently been reported[36]. Of note, in our rural population the effect of maternal work in stables during pregnancy on the child's atopy risk was independent from the mother's smoking habits. The lack of insight into the determinants of the increase in the prevalence of asthma and allergies over time might therefore in part be explained by the neglect of the potential effect of preconceptional exposures of the parents.

In conclusion, our results suggest that maternal exposures to environments rich in microbial exposures determine the priming of a child's immune response. For organ-specific manifestation of allergic diseases, additional factors, continuing exposure, or both effective later in life seem to be important. Our findings stress the importance of the identification of the appropriate time window leading to the modulation of immune responses and disease manifestation, respectively. Longitudinal studies will eventually deepen our understanding of the relevant temporal sequences.

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CHAPTER 7

Early-life exposure to microbial components induces persistent modulations of the innate immune system in mice

Written as manuscript for publication

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Abstract

Prenatal exposure to microbial components is associated with protection against allergies in school age children. Furthermore, it prevents subsequent allergen-induced sensitization and airway inflammation in mice and it induces the expression of Toll-like receptor genes, the principal pattern recognition receptors of the innate immune system. How long the changes in the innate immune system persist after exposure to microbes in early life is unknown. In this study, we assessed that a single challenge of newborn mice with a mixture of S.aureus and E.coli was able to enhance the gene expression of marker genes of the innate immune system persistently over three months without induction of chronic inflammation. These results indicate that the innate immune system may have a memory-like character.

Introduction

The innate immune system is an evolutionary conserved system controlling infections in an early phase. Vertebrates improved the immune response by evolving the more effective adaptive immunity consisting of an enormously diverse antigen-recognizing lymphocytes pool[1]. After infection lymphocytes, bearing receptors able to bind antigens, start to clonally expand and to undergo hypermutations and affinity maturation. With the end of the infection, most of the antigen-specific lymphocytes die but the adaptive immunity maintains the knowledge how to fight the infection through the generation of antigen-experienced memory cells. This pool of long-lived memory cells is continuously growing and guarantees a more rapid and robust response to subsequent encountering of a previously experienced antigen[2].

The innate immune system is generally characterized by missing memory. It recognizes conserved foreign patterns by germ-line encoded receptors. Toll-like receptors (TLR) constitute the principal family of these receptors[3]. Ligand binding to TLR triggers a tightly regulated signaling cascade inducing defence mechanisms, co-stimulatory molecules, and cytokines necessary for activation of the adaptive immunity[4, 5]. Recent studies showed that the human innate immune systems adapts to exposure to foreign structures, too. Expression of TLR and of regulatory molecules like suppressor of cytokine signaling (SOCS)-1 were induced by environmental exposure to microbial components[6]. Especially prenatal or neonatal exposure of children seems to shape the innate immune system[7]. These modulations are associated with reduced inflammatory responses and protection against allergy and autoimmunity[8, 9].

In this study we were interested whether the innate immune system has memory-like character, too. We investigated whether singular application of microbial components to newborn mice establishes persistent memory in the innate immune system. Our mouse model

showed that expressions of TLR genes, of genes of TLR signaling cascade, and of genes of molecules inducing T cell independent immunoglobulin isotype switching were elevated three months after application of microbial components. These enhanced modulations were not based on chronic inflammation.

Methods

Mice. We purchased pregnant C57BL/6 mice (Harlan) and kept them under optimal husbandry conditions. Two, four, and six days after birth, we injected the newborn mice subcutaneously in the neck either with 30µl of a 0.9% NaCl solution, 30µl of a heat-killed (60°C, 1 hour) mixture of 50 thousand Staphylococcus aureus (S. aureus) and 50 thousand Escherichia coli (E. coli) bacteria, or 30µl of a 1:1 dilution of complete Freund's adjuvant (CFA; Sigma) using a BD Micro-Fine Insulin syringe (0.5ml, 0.30mm (30G) x 8mm; BD biosciences). Three months after the last injection, we obtained blood plasma and lymph nodes (superficial cervivals, axillary, and brachial). We added 500µl RNAlater (Ambion) solution to lymph nodes, shock-froze them in liquid nitrogen, and stored them at minus 80°C. We took full blood with a syringe out of the heart after opening the thorax and protected it against coagluation with 100µl heparin. The plasma was separated by centrifugation.

Tissues homogenization and RNA extraction. We transferred 30mg of the thawed lymph nodes to RLT buffer (QIAmp RNA Blood Mini Kit, Qiagen) supplemented with 1% β-mercaptoethanol and homogenized it using a Retsch MM300 mixer mill (Retsch). For total RNA extraction, we used the QIAmp RNA Blood Mini Kit (Qiagen) supplemented with RNase-free DNase (Qiagen).

RT-PCR and quantitative real-time PCR. For reverse transcription (RT) of RNA we used 300ng of total RNA in a final volume of 30µl and added adequate amounts of TaqMan® Reverse Transcription Reagents (Applied Biosystems). Quantitative real-time PCR was performed in an ABI Prism 7900 Sequence Detection System™ (Applied Biosystems) using the TaqMan® low density array system of Applied Biosystems. 18S rRNA, glucuronidase beta (GUS), and beta-2-microglobulin (B2M) acted as endogenous controls. To each fill port we added 100µl of a mixture of cDNA (100ng) and TaqMan Universal PCR Master Mix™ (Applied Biosystems). The determined gene expression values were normalized to the geometric mean of the parallel measured endogenous controls 18S rRNA, GUS, and B2M[10]. We analyzed the data with the comparative Ct method according to the manufacturer's instructions (Applied Biosystem).

Immunoglobulin quantification. We determined levels immunoglobulin isotypes in untreated plasma samples of mice using the Beadlyte mouse immunoglobulin isotyping kit

(Upstate) and analyzed it with a Luminex instrument (Luminex Technology). The amount of immunoglobulin was related to the total amount of protein in the plasma measured with a BCA^{TM} protein assay kit (Pierce).

Results

To assess a memory of the innate immune system we challenged newborn mice with a cocktail of heat-killed S.aureus and E.coli bacteria. Furthermore, we stimulated newborn mice with complete Freund's adjuvant (CFA). This mixture of mycobacteria and mineral oil is known to have long-term immuno-stimulatory effects[11]. Three months after application, we determined the expression of TLR genes and of genes of the TLR signaling cascade in lymph nodes situated near the application area by quantitative real-time PCR (Figure 1). We were able to detect significant up-regulation of TLR2, TLR3, and TLR4, and of the signaling molecule MyD88 through application of the E.coli/S.aureus mixture, while CFA failed to induce marker genes of the innate immune system. Furthermore, a slight induction of CD14 and IRAK-4 expression was observed but the expression of TLR5, TLR7, TLR9, and of other genes of signaling molecule failed to alter (data not shown).

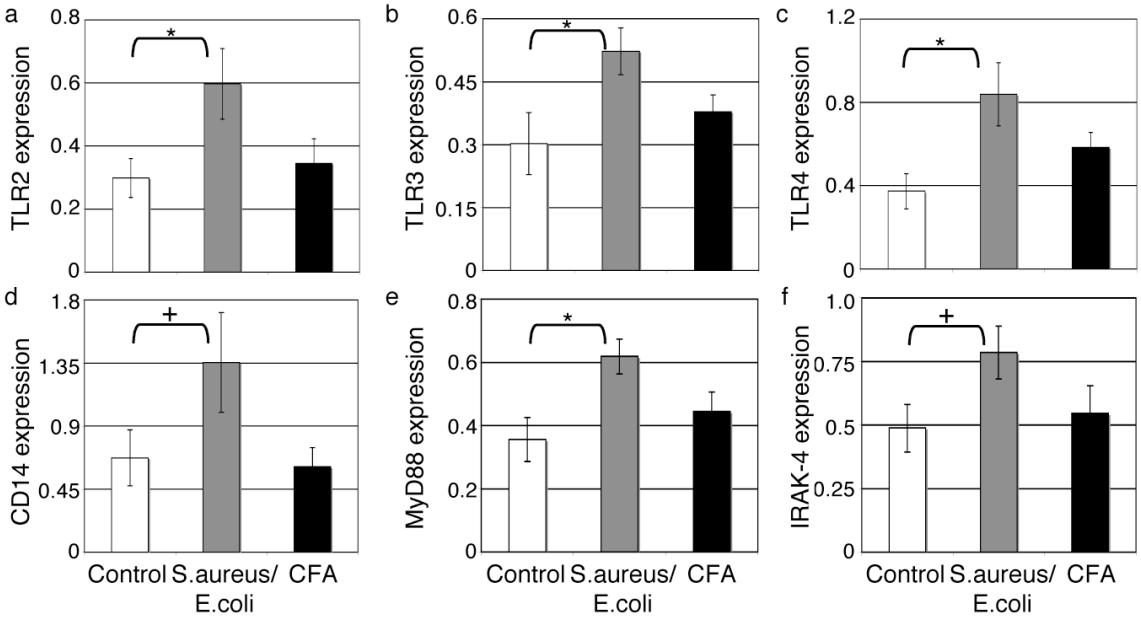

FIGURE 1. Expression of maker genes of the innate immune system in mice three months after challenging them with bacterial components. We compared expression of TLR genes (a-c), of CD14 gene (d), and of genes of TLR signaling cascade (e, f) in lymph nodes of mice treated with a mixture of S. aureus and E.coli (grey bars) or with CFA (black bars) three months ago. Control mice were treated with NaCl solution (white bars). All expression values were normalized to the geomean of three endogenous controls. Bars represent the mean of 8 mice; error bars are standard errors of the mean; * = $p < 0.05$, + = $p < 0.1$.

Next, we were interested whether application of bacterial components had also long-term consequences on the function of the innate immune system. Since a principal function of the innate immune system is to release cytokines, we measured the expression of cytokine genes in lymph nodes of stimulated mice by quantitative real-time PCR (Figure 2). The expression of TNF gene and to a minor extant the expression of interleukin (IL)-6 gene were still elevated three months after application of S.aureus and E.coli, while CFA had no effects. Furthermore, the gene expression of IL-12, gamma interferon (IFN-γ), IL-13, IL-10, and TGF-β was unaffected (data not shown).

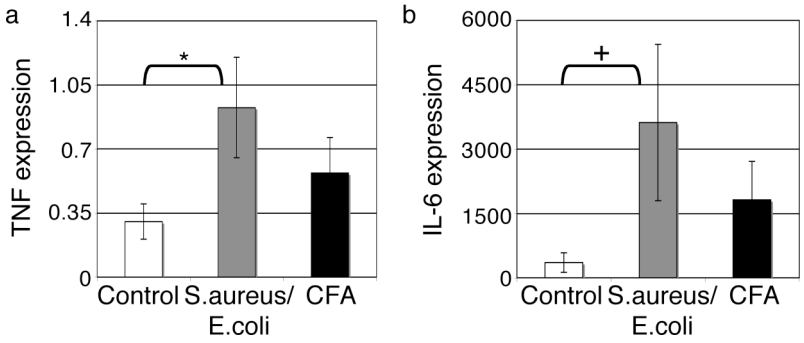

FIGURE 2. Expression of TNF and IL-6 in mice stimulated with bacterial components three months ago. We compared the gene expression of TNF (a) and IL-6 (b) in lymph nodes of mice three month after they were challenged with a mixture of S. aureus and E.coli (grey bars), with CFA (black bars), or with NaCl (control, white bars). All expression values were normalized to the geomean of three endogenous controls. Bars represent the mean of 8 mice; error bars are standard errors of the mean; * = $p < 0.05$, + = $p < 0.1$.

Another function of the innate immune system is to direct T helper cell differentiation. To assess whether mice challenged with bacterial components had altered T helper cell differentiation, we measured the expression of T helper cell marker genes in lymph nodes by quantitative real-time PCR (Figure 3). S.aureau and E.coli enhanced the expression of the T helper cell type (Th)-1 marker T-bet of borderline significance. The expression of the Th-2 marker GATA-3 and of FOXP3, a marker for T regulatory (Treg) cells, was slightly increased but non-significant. CFA failed to alter the expression of T cell markers.

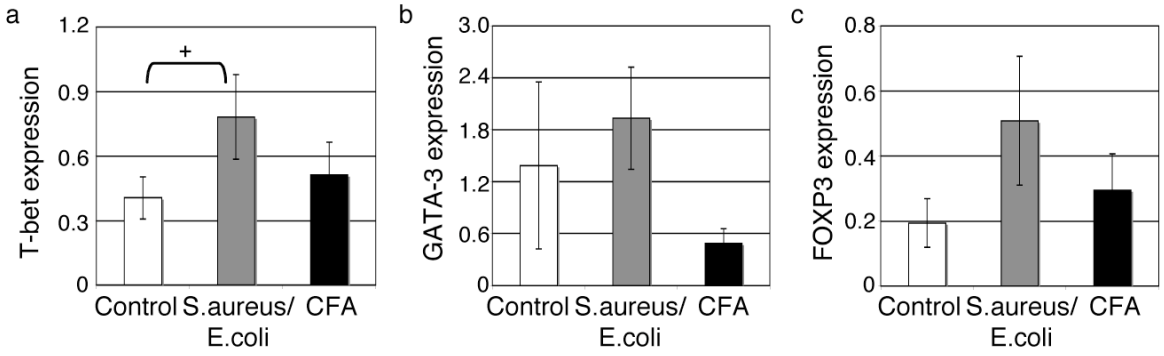

FIGURE 3. Expression of T helper cell and T regulatory cell marker in mice stimulated with bacterial components three months ago. We compared the expression of T-bet (a), GATA-3 (b), and FOXP3 (c) gene in lymph nodes of mice stimulated with a mixture of S. aureus and E.coli (grey bars), with CFA (black bars), or with NaCl (control, white bars) three months ago. All expression values were normalized to the geomean of three endogenous controls. Bars represent the mean of 8 mice; error bars are standard errors of the mean; * = $p < 0.05$, + = $p < 0.1$.

Innate immunity has the potential to induce T cell independent immunoglobulin isotype switching via release of BAFF (B lymphocyte stimulator protein) and APRIL (a proliferation-inducing ligand). BAFF and APRIL bind B cells directly. We assessed the expression of BAFF and APRIL genes by quantitative real-time PCR in lymph nodes of mice treated three months ago with bacterial components (Figure 4). We found that S.aureus and E.coli induced the expression of the BAFF gene and also of the APRIL gene but less significant. By contrast, expression of CD40 ligand, a marker for T cell dependent immunoglobulin isotype switching, was unaltered. CFA failed to induce expression of any of these molecules.

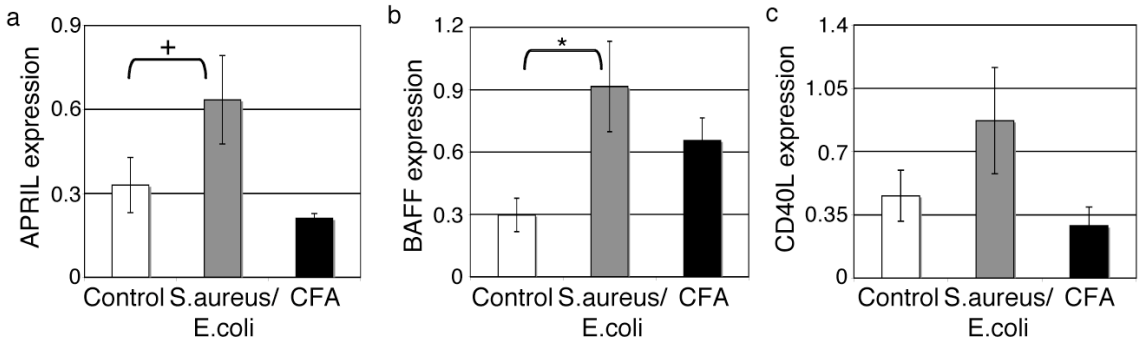

FIGURE 4. Expression of ligands inducing T cell independent or T cell dependent immunoglobulin isotype switching in mice stimulated with bacterial components three months ago. We challenged newborn mice with a mixture of S. aureus and E.coli (grey bars), with CFA (black bars), or with NaCl (control, white bars) and compared the expression of the APRIL (a), BAFF (b), and CD40 ligand (c) gene in lymph nodes three months later. All expression values were normalized to the geomean of three endogenous controls. Bars represent the mean of 8 mice; error bars are standard errors of the mean; * = $p < 0.05$, + = $p < 0.1$.

Chonic inflammation could be the cause for enhanced activiation of the innate immune system and thereby alter the expression of genes. Because S.aureus and E.coli enhanced expression of many genes of the innate immune system, we were interested whether application of bacterial components induced chronic inflammation. To investigate this, we measured immunoglobulin levels in blood plasma of mice as marker for systemic inflammation using Luminex technology (Figure 5). S.aureus and E.coli application failed to enhance immunoglobulin secretion, while CFA enhanced immunoglobulin levels of all assessed isotypes.

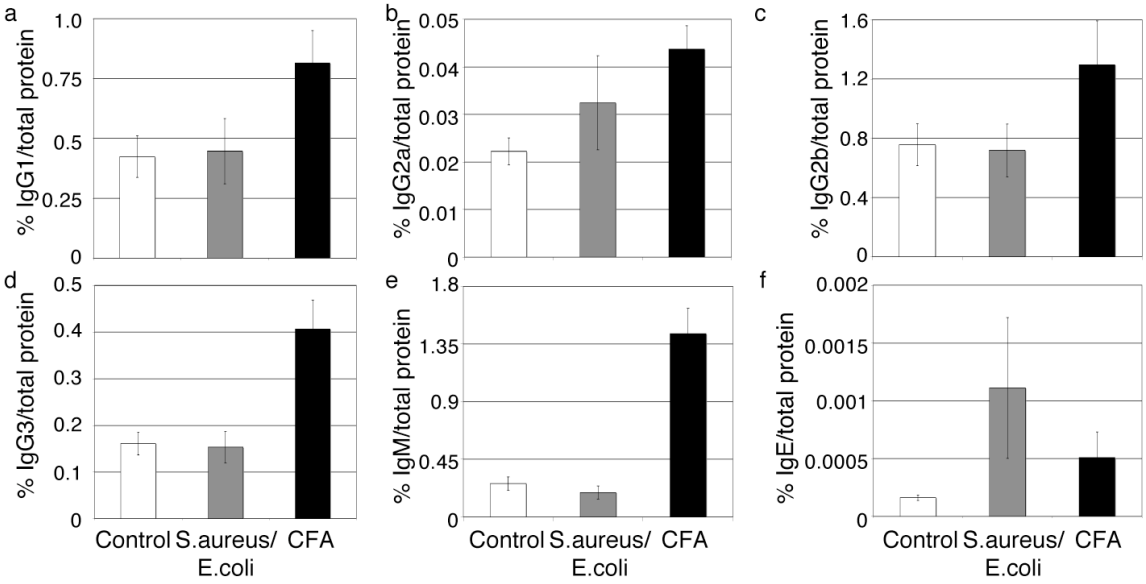

FIGURE 5. Comparison of immunoglobulin levels in blood plasma of mice treated with bacterial components three months ago. We applied a mixture of S. aureus and E.coli (grey bars), CFA (black bars), or NaCl (control, white bars) to newborn mice and assessed immunoglobulin levels in blood plasma three months later. All immunoglobulin levels are expressed as percentage of total protein in the blood plasma. Bars represent the mean of 8 mice; error bars are standard errors of the mean.

Discussion

Three things feature the memory of the adaptive immune system. It is generated through encountering of foreign antigens, it is long lived, and it has functional consequences achieved by faster cell proliferation and enhanced secretion of immunoglobulin via subsequent encountering of a previously experienced antigen. Our study indicates a memory of the innate immune system, too. We observed that single encountering of microbial components shapes the innate immune system in a persistent way as observed by elevated expression of various marker molecules. Remarkable, these modulations were not induced due to chronic inflammation. By contrast, CFA inducing a broad chronic inflammation failed to have persistent effects on innate immunity.

The concept of our study failed to answer whether these modulations have functional consequences. Our data indicate a slight shift towards Th-1 differentiation and enhanced T helper cell independent immunoglobulin isotype switching shown by increased expression of BAFF and APRIL. Data of epidemiologic and animal studies indicate that modulations having similar character to the ones we observed in our study, have the potential to protect against inflammatory, allergic, and autoimmune diseases[5-7, 9, 12, 13]. Therefore, we propose that the innate immune system has a memory function.

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CHAPTER 8

Discussion and Outlook

An allergic immune response is an inappropriate or an overreaction of the immune system against harmless pathogens. Besides genetic predisposition, environmental exposure to various factors such as microbes, allergens, air pollution, or dietary factors influence the development of allergies[1]. A very illustrative example, how genetic predisposition and environmental factors collude, was the CD14 polymorphism (CD14/-1721). Consuming non-pasteurized milk protected only children carrying the AA and less extended the AG allele against allergic disorders. Furthermore, the AA and the AG allele correlated with enhanced CD14 gene expression due to non-pasteurized milk consumption (Bieli et al., submitted).

The hygiene hypothesis has been postulated as an explanation for the increased prevalence of allergic disorders in Western society[1]. In this work, we investigated how environmental factors influence the immune system in context of the development of allergic diseases. We used farmers' children as model because rural lifestyle turned out to be highly protective[2-4]. The innate immune system constitutes the first contact to invading microbes and drives the adaptive immune response by T helper cell activation. Therefore, it has been proposed that the innate immune system has a crucial role in protecting against the development of allergies[5]. Support came from results that TLR gene expression was induced in farmers' children and several polymorphisms in genes of the innate immunity associated with allergic diseases[1, 6-10]. The widespread immunological basis of the hygiene hypothesis is that reduced microbial stimulation of TLR of innate immune cells in early life leads to a shift towards Th-2 responses against allergens[11]. Besides of epidemiological data describing an increase of Th-1 and Th-2 associated disease in Western society[12], our gene expression measurements failed to show an enhanced Th-1 response in farmers' children. Furthermore, the recently postulated Treg cells were not enhanced in these children. Therefore, reduced hygiene conditions of a farm failed to influence T cell differentiation and they do not constitute the immunological basis of the hygiene hypothesis.

All T helper cell phenotypes were associated with immunoglobulin class switching to IgE in children. Therefore, elevated T helper cell levels, probably generated by genetic predisposition or by unknown environmental conditions, enhance immunoglobulin CSR to IgE and seem thereby to support the development of allergies. Skewing of the T helper cell balance may only become important if microbial levels are much higher, as it is the case during an infection.

An innate immune system, shaped by reduced hygienic conditions of a farm, is able to decrease the risk to develop allergies through reduction of inflammatory responses and of immunoglobulin CSR to IgE. Regulation of the innate immune system has to be very tight. Insufficient activation leads to inefficient defence against an infection, over-activation may lead to chronic inflammation, allergy, and autoimmunity. In our epidemiological studies, we

observed that expression of the regulatory molecule of the TLR signaling cascade SOCS-1 was influenced by exposure to environmental microbial components. Enhanced expression of these molecules shift the innate immune system into a status of tolerance meaning that it exhibits decreased inflammatory responses to further applications of microbial components. But not all of these regulatory molecules seem to have the same relevance in induction of tolerance mediated by exposure to environmental microbes in children. We failed to reproduce this function for ST2. ST2 is up-regulated shortly after administration of bacterial components and may regulate TLR signaling cascade in an early phase[13, 14]. Chronic exposure to microbes, as it was the case for the children of our study, reduced the ST2 expression. This, in turn, was associated with reduced non-phenotype specific T helper cell activation and reduced incidence of allergies. Therefore, ST2 may exhibit its protective function against allergies via regulating T helper cell activity, which we showed to be crucial in inducing immunoglobulin CSR to IgE.

Children, exposed to microbes in the environment, build up a network of molecules desensitizing the innate immune system against harmless antigens or antigens in low-dose. This network has the potential to inhibit an inappropriate adaptive immune response, as it is the case in allergic disorders. The expression of SOCS-1 correlates with reduced IgE levels in serum and expression of TLR and BAFF negatively associates with immunoglobulin CSR to IgE. The expression of these molecules, in turn, is enhanced via exposure to environmental microbes. Whether these immunological mechanisms are linked together or whether there are other regulatory molecules involved, for example the MHC class II molecule, which we described to have regulatory functions in the innate immune system, is going to be the subject of further data analysis. To assess the complexity of the complete regulatory network of the innate immune system, alternative methods for data analysis may be used. Biclustering[15, 16], chaos theory, or dynamic models are a few examples. Furthermore, it would be interesting to investigate whether an innate immune system shaped via exposure to environmental microbes protects against the development of autoimmune diseases, too. Especially, the pattern of natural antibodies may be influenced via exposure to different environmental conditions. Natural antibodies are germ-line encoded antibodies binding almost exclusive self molecules. Although the function of natural antibodies is not clear, specific self molecules recognized by these autoantibodies appear to form clinically defining patterns heralding for example autoimmune diseases[17].

Epidemiological studies are a useful tool to investigate immunity in context of environmental conditions because these conditions can hardly be simulated in the lab. The option to check the relevance of mechanisms discovered *in vitro* or with mouse models in real-life conditions is the main advantage of such systems. Not all results achieved by lab conditions have relevance in normal life as our results of the Th-1/Th-2 balance showed.

Drawbacks of epidemiological studies are the impreciseness in questionnaires and the immense time and effort. To assess significant correlations between parameters, generally huge numbers of samples are necessary to exclude interfering effects. Nevertheless, this work shows that strong and promising correlations can be found in small populations.

How long children have to live in a rural environment until protection against the development of allergic disorders establishes was another aspect, we investigated in this work. Children of the PARSIFAL study were highly protected if their mother had contact to stable or stable animals during pregnancy[7]. Furthermore, we achieved persistent elevation of the expression of marker genes of the innate immune system via single administration of microbial components in early life of mice and thereby protected them against the development of allergies[18]. Therefore, it seems that prenatal or early life exposure to microbes is crucial to induce a memory-like function of the innate immune system that may be able to protect against allergies. Interesting new insights in this field is going to give us the PASTURE (Protection against Allergy: Study in Rural Environments) study. For this longitudinal epidemiological study, pregnant woman living on farms were recruited. Newborn children spend blood at their birth (cord blood), at the age of one, and at the age of 5 years. Therefore, we are able to observe the development of protective mechanisms through exposure to environmental microbial components over time. Furthermore, we are going to investigate whether long-term modulations of innate immune system are based on antigen-persistence in the body as it is proposed for the memory of the adaptive immunity or due to changes in cytosine methylation patterns of gene promoters[19, 20]. Cytosine methylation within CpG dinucleotides of DNA acts in concert with other chromatin modifications to maintain specific genomic regions in a transcriptional silent state.

Our data support the idea of a vaccination-like therapeutic challenge of the innate immune system. Currently, promising clinical studies with probiotic microbes are ongoing. Prenatal administration of probiotic microbes to children had preventive effects on atopic diseases at least until the age of 4 years. Probiotic microbes are live microorganisms culturing the gut and thereby providing a beneficial stimulus to the innate immune system. Interestingly, porbiotic microbes failed to reduce antigen-specific IgE antibodies[21]. Challenging the innate immunity of mice by TLR agonists provided interesting results, too. Application of TLR2, TLR4, and TLR9 ligands reduced the degree of allergic asthma through reduction of inflammation, total serum IgE and number of T helper cells in the lung[1, 18, 22]. Another promising therapeutic target in the prevention of allergic disorders is the use of non-pathological helminth parasites, since they have high protective effects although they induce a large Th-2 response. The results of the currently running epidemiological studies are going to show whether helminth eggs are useful as vaccination against allergic disorders. It would be interesting to investigate

whether these therapeutic tools have beneficial effects on the regulatory network of the innate immunity.

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Abbreviations

AICDA	activation-induced cytidine deaminase
ALEX	Allergy and Endotoxin
APC	antigen-presenting cells
APRIL	a proliferation-inducing ligand
BAFF/BLyS	B lymphocyte stimulator protein
BAFF-R/BR3	BAFF receptor
BCL	B cell lymphoma
BCMA	B cell maturation antigen
BLP	bacterial lipoprotein
CIITA	class II transactivator
CARD	caspase activation and recruitment domain
CD40L	CD40 ligand
CFA	complete Freund's adjuvant
CSR	class-switch recombination
CTL	cytotoxic T lymphocyte
CTLA	cytotoxic T lymphocyte antigen
DC	dendritic cells
ERK	extracellular-signal-regulated kinase
EU	endotoxin units
FADD	Fas-associated via death domain
GLT	germ-line transcript
hBD	human β-defensin
HEK	human embryonic kidney
ID	inhibitor of DNA binding
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin

INF	interferon
IRAK	IL-1R-associated kinase
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motif
JNK	Jun N-terminal kinase
ko	knock-out
LPS	lipopolysaccharides
LRR	leucin-rich repeats
Mda	melanoma differentiation associated gene
MCD	methyl-β-cyclodextrin
MHC	major histocompatibility complex
MyD88	myeloid differentiation primary-response protein 88
NOD	nucleotide-binding oligomerization domain
PAMP	pathogen-associated molecular pattern
PARSIFAL	Prevention of Allergy Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle
PASTURE	Protection against Allergy: Study in Rural Environments
PBL	peripheral blood leukocytes
PI3K	phosphatidylinositol 3-kinase
PKR	dsRNA-dependent protein kinase
PRR	pattern recognition receptors
RAG	recombination-activating genes
RIG	retinoic-acid-inducible protein
RIP	receptor-interacting protein
RSS	recognition signal sequences
SARM	steril α and armadillo motifs
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
TAB	TAK1 binding proteins

TACI	transmembrane activator and calcium modulator and cyclophylin ligand interactor
TAK	TGF-β-activated kinase
TCR	T cell receptor
Th	T helper cell
TIR	Toll/IL-1R homology
TIRAP/MAL	TIR-associated protein
TLR	Toll-like receptors
TNF	tumor necrosis factor
TOLLIP	Toll-interacting protein
TRAF	TNFR-associated factor
TRAM	TRIF-related adaptor molecule
Treg	T regulatory cell
TREM	triggering receptors expressed by myeloid cells
TRIF/TICAM1	TIR-domain-containing adaptor protein-inducing IFN-β
wt	wild type

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Curriculum vitae

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Additional publications and manuscripts

- Gerhold K, Avagyan A, Seib C, Frei R, Steinle J, Ahrens B, Dittrich AM, Blumchen K, Lauener R, Hamelmann E. *Prenatal initiation of endotoxin airway exposure prevents subsequent allergen-induced sensitization and airway inflammation in mice.* J Allergy Clin Immunol. 2006 Sep;118(3):666-73.

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- Weber-Chrysochoou C, Steinle J, Tiner E, Loeliger S, Frei R, Hamelmann E, Lauener RP. *Induction of Acidic Mammalian Chitinase, airway hyperreactivity and inflammation caused by fungi in non-allergic mice*. Manuscript in preparation.

Congress contributions

- Triangel-Meeting, Zurich, 2004: Oral presentation

- EAACI-GA2LEN Davos Meeting Basic Immunology Research in Allergy and Asthma, Davos, 2005: Oral presentation

- Swiss Society of Immunology (SGAI/SSAI), Bern, 2005: Poster presentation

- World Allergy Congress (WAC) / European Academy of Allergy and Clinical Immunology (EAACI), Munich, 2005: Poster presentation

- Applied Biosystems Gene Expression Seminar, Zurich, 2005: Oral Presentation

- Triangel-Meeting, Berlin, 2006: Oral presentation

- Swiss Society of Immunology (SGAI/SSAI), Zurich, 2006: Poster presentation

- European Academy of Allergy and Clinical Immunology (EAACI), Vienna, 2006: Poster presentation

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